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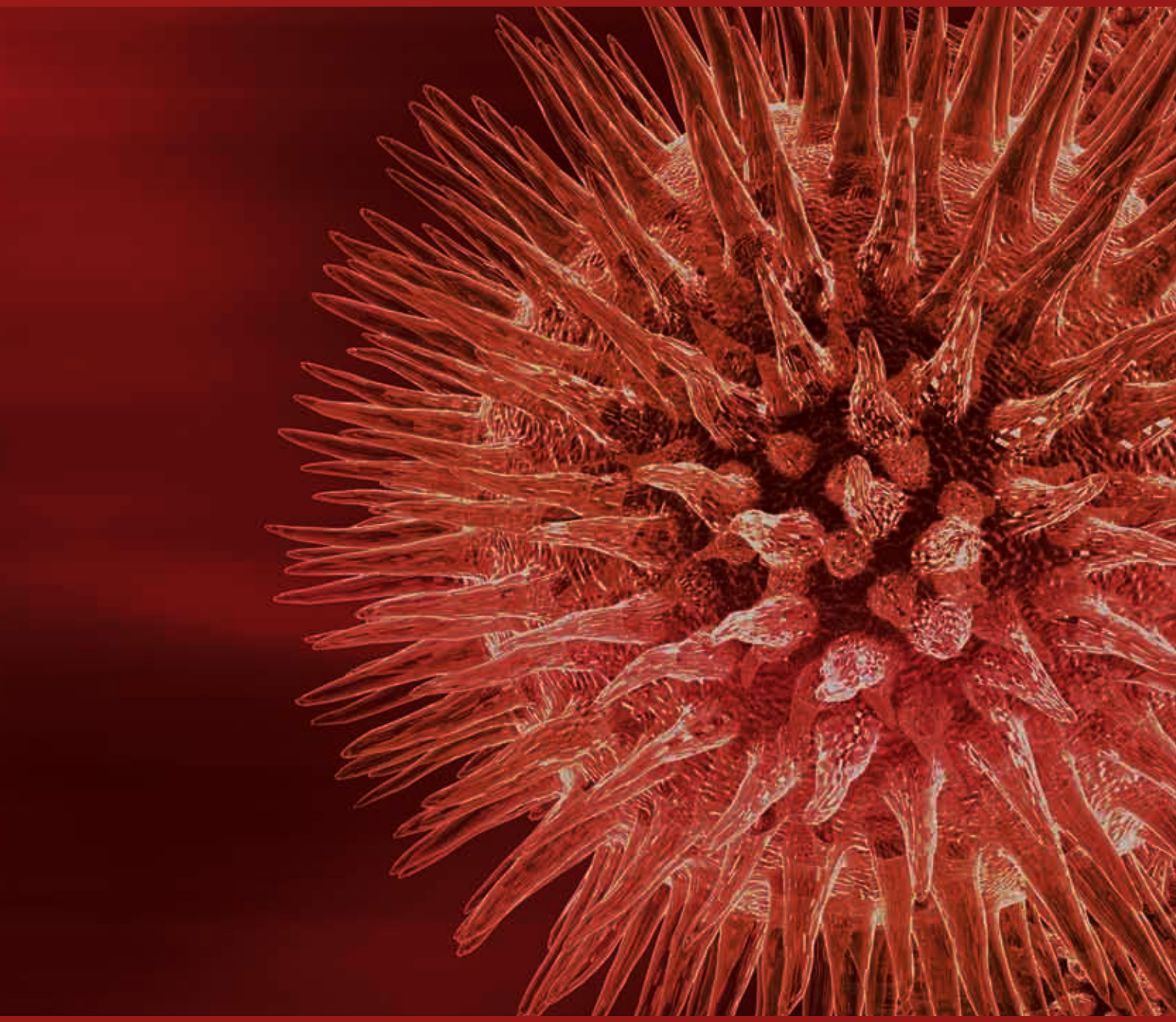
Gene-Gene Interaction in Maternal and Perinatal Research

Guest Editors: Janet S. Sinsheimer, Robert C. Elston, and Wenjiang J. Fu



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Editorial

Gene-Gene Interaction in Maternal and Perinatal Research

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Recent genomic research increases our understanding of the causes of complex diseases and strengthens the evidence that many complex diseases, even those with late age of onset, are caused in part by genetically induced, adverse prenatal environments. This special issue of the Journal of Biomedicine and Biotechnology specifically examines the evidence that gene-gene interactions during early development influence human traits and diseases.

Mothers largely determine the fetal environment; therefore the maternal genotypes are expected to influence fetal development. Two types of gene-gene interactions are possible during pregnancy, intragenerational interaction, and intergenerational interaction. Intragenerational effects concern gene-gene interactions within an individual's genome to affect their own disease outcome. Intergenerational effects lead to conflicts between a mother and her fetus in some cases and, in other cases, they lead to beneficial environments that protect against disease. Intergenerational interactions can occur between genes in a child's genome affecting the mother's phenotype or between genes in a mother's genome affecting the child's phenotype. There can also be interactions between the maternal genes and fetal genes, such as maternal-fetal genotype incompatibility, that cause changes in the mother's or the child's phenotype.

Maternal-fetal genotype (MFG) incompatibility is not just a theoretical possibility. The most well-known example of this form of gene-gene interaction is Rhesus factor D-induced hemolytic disease of the newborn [1], in which a Rhesus-negative mother develops antibodies to her Rhesus-positive fetus, leading to destruction of fetal red blood cells. Gene-gene interactions have also been studied as causes of pregnancy complications such as gestational hypertension and diabetes [2], but they have rarely been studied as risk factors beyond pregnancy complications. MFG incompatibility, in particular, has been reported to play an important role in the development of a number of disorders, including preeclampsia, preterm delivery, small-for-gestational-age neonates, and schizophrenia. Although genetic conflict has been postulated as playing a role in these disorders, rigorous modeling and quantitative analysis of these gene-gene interactions have only begun recently, as a result of biotechnological developments that have made it possible to conduct these investigations.

Researchers conducting genome-wide association studies (GWAS) occasionally test for intragenerational gene-gene interactions, but they seldom test for maternal genetic influences on the phenotypes—and almost never test for intergenerational gene-gene interactions. There are several reasons for these omissions. First, testing for maternal genetic influences requires that both mothers and children be genotyped. Very often, the father's genotype is also required by numerous analytical methods. This requirement increases the number of individuals who must be genotyped and also increases the number of tests conducted, which makes the study expensive. Second, it may even be impossible to obtain maternal genotypes if the disease has an adult onset.

It is our contention that limiting gene discovery to single-gene analysis using unrelated individuals is a mistake. With much of the genetic architecture of complex traits left unexplained, more investment into intergenerational gene-gene interactions is warranted. The evidence for multiple genetic influences during early development takes on a variety of forms and the eight articles chosen for the special issue reflect this variety.

Lupo et al. [3] examine gene-gene interactions in folate metabolic genes as risk factors for relatively common birth defects such as conotruncal heart defects (CTHDs), following up on their earlier work showing that some polymorphisms in folate metabolic genes are associated with CTHDs when these polymorphisms are analyzed separately [4]. In the current paper, they not only test for child gene-gene interactions, but also for maternal gene-gene interactions. Interestingly, their most significant results are observed for a maternal gene-gene interaction between CBS844ins68 and rs1801133. Neither locus is significantly associated in the marginal analysis of maternal effects. Given that they also observe child allelic effects, it would be interesting to know whether there are any maternal-fetal genotype incompatibilities.

It is natural to wonder just how widespread intergenerational gene-gene interactions are. Is it reasonable to suggest that maternally expressed genes or interactions between mother's and child's genes represent a substantial portion of the attributable risk of complex disease? Priest and Wade [5] use evolutionary population genetic theory to tackle this question. Their simulations of two unlinked loci, one expressed maternally and the other expressed in the child, demonstrate that maternally expressed alleles that increase disease susceptibility in the child can have higher frequencies and persist longer than child expressed alleles that increase disease susceptibility. Gene-gene interactions between maternal and fetal genes can lead to even greater frequency and persistence of these fitness-reducing alleles. They provide some intuition for these results by pointing out that deleterious genes that are expressed maternally hide out unexpressed in fathers, and are thus partially shielded from purifying selection. Their results also show that maternally expressed genes and maternal-fetal gene interactions will be difficult to detect in typical linkage disequilibrium mapping study designs like GWAS.

Many of the analytical methods designed to test for maternal allelic effects or MFG incompatibility rely on an assumption of mating symmetry in the population. Mating asymmetry and maternal allelic effects can be confounded in these methods [6], so it is important to have an analysis method that is robust to mating symmetry violation when mating asymmetry is suspected in the population. By comparing methods through simulation, Healy et al. [7] find that the case-triad/case-control hybrid test performs well under mating asymmetry. They then apply the test to examine the association of SNPs in the promoter of cell-cycle genes with childhood pre-B acute lymphoblastic leukemia to find both maternal allelic and child allelic effects.

Healy et al. [7] do not directly test for maternal-fetal genotype incompatibility. This test requires modifying the underlying model of the case-triad/case-control hybrid test to allow for maternal-fetal genotype interaction. In their analysis of the association of TNF-α G308A polymorphisms with preterm delivery (PTD), Liang et al. [8] make the necessary modifications to a hybrid case-parent trio and control-parent model to allow for MFG incompatibility. Rather than limiting the analysis to a specific form of MFG incompatibility such as HLA matching [6] or noninherited maternal antibody effects [9], they model joint maternal and child allelic effects, which requires 6 parameters for a diallelic locus. The maternal and fetal main effect model requires only 4 parameters, so the null hypothesis of no MFG incompatibility can be tested with a likelihood ratio test that compares these two models without specifying the MFG mechanism. The disadvantage of their approach is a loss of power when the MFG incompatibility mechanism is known and requires fewer parameters (such as the case for RHD incompatibility).

The effects of maternal-fetal genotype incompatibility are not limited to neonatal, or even childhood, disorders. In her review article, Palmer [10] summarizes the evidence that MFG incompatibilities are risk factors for schizophrenia. She also provides compelling biological arguments to support the viewpoint that RHD and HLA-B incompatibilities are consistent with the neurodevelopmental hypothesis of schizophrenia. Interestingly, the two MFG incompatibilities, which have different mechanisms of action but ultimately may be risk factors because they both can lead to hypoxia, show different sex effects. RHD incompatibility has a stronger effect in males than females and HLA-B incompatibility has a stronger effect in females than males. Palmer [10] points out that olfactory deficits in the parents of schizophrenics may play a role in HLA-B incompatibility, and this could certainly be true. Olfactory deficits produce mating-type frequencies for the parents of cases that are different from the mating-type frequencies for the parents of controls. The MFG test is independent of the mating-type frequencies of controls [6] and so cannot be used to test this hypothesis. The significant results in [11], however, cannot be explained by this gene-environment covariation alone [12] because they are the consequence of mating asymmetry to a lesser extent and of transmission distortion to a greater extent [6, 9, 11]. Olfactory deficits in the parents should not lead to transmission distortion.

Developmental effects on adult onset, complex disease are often far from simple in their mechanism. Perinatal environmental effects can be highly variable depending on the child's genetic makeup. The correlation of birth weight to adult blood pressure has been much studied, but the reasons for the association are unclear. Using data from the Bogalusa Heart Study, a longitudinal prospective study documenting cardiovascular risk factors, Chen et al. [13] determine that the degree of association between birth weight and age-related trends in diastolic and systolic blood pressure is dependent on polymorphisms in beta-adrenergic receptor genes by finding significant three-way interactions of β_2-AR ARG16GLY, β_3-AR TRP64ARG, and birth weight. Interestingly, the main effects of these genes and their two-way interactions are not significant.

The effects of maternal fetal genotype incompatibilities must be modulated by prenatal environment. As an example, maternal infections during pregnancy have been implicated as risk factors for disorders in children as diverse as congenital abnormalities [14], hearing loss [15], and schizophrenia [16, 17]. Indeed there may be gene-by-maternal urinary tract infection effects in schizophrenia [18]. Thus it is

important to understand the genetic risk factors underlying recurrent urinary tract infection (UTI). Zaffanello et al. [19] summarize the research into the genetic determinants of UTI and find that, of the candidate genes studied, only HSPA1B, CXCR1, CXCR2, TLR2, TLR4, and TGF-β1 are significantly associated with recurrent UTI. Of these genes, CXCR1 is the most extensively studied and supported. CXCR1 encodes the receptor for the IL-8 chemokine [20], and chemokines are an important part of the inflammatory process. The research into the genetic basis of recurrent UTIs has only just begun, so more work is needed to determine whether any of these 6 genes interact intra- or inter-generationally.

Nowhere is the complex interplay of maternal and fetal genetics more intriguing than for phenotypes originating with the placenta. Hoegh et al. [21] profile gene expression of placenta tissue from women affected with preeclampsia and compare this gene expression profile to the gene expression profile of placenta tissue from women whose pregnancies were normal. Due to the relatively small sample size, their study may be limited to those genes that show large differences in expression; however, they still find 21 differentially expressed genes that can be classified into a number of roles, including placentation, oxidative stress, inflammatory response, and blood pressure regulation. Their results and earlier work implicating joint maternal and fetal risk factors [22] suggest that a complex network of concerted maternal-fetal gene actions is responsible for preeclampsia.

Research into methods to detect maternal and perinatal gene-gene interactions and to understand the mechanisms of these interactions is just beginning. The articles in this special issue represent a broad range of approaches and each one provides additional evidence that these gene-gene interactions play a significant role in human disease. More research is needed, however, to determine just how great an impact maternal and perinatal gene-gene interactions have in determining human phenotypes. It is hoped that a thorough understanding of maternal and perinatal gene-gene interaction will lead to major breakthroughs in prevention, treatment, and therapeutics.

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Wenjiang J. Fu

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Research Article

Gene-Gene Interactions in the Folate Metabolic Pathway and the Risk of Conotruncal Heart Defects

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Conotruncal and related heart defects (CTRD) are common, complex malformations. Although there are few established risk factors, there is evidence that genetic variation in the folate metabolic pathway influences CTRD risk. This study was undertaken to assess the association between inherited (i.e., case) and maternal gene-gene interactions in this pathway and the risk of CTRD. Case-parent triads ($n = 727$), ascertained from the Children's Hospital of Philadelphia, were genotyped for ten functional variants of nine folate metabolic genes. Analyses of inherited genotypes were consistent with the previously reported association between *MTHFR* A1298C and CTRD (adjusted $P = .02$), but provided no evidence that CTRD was associated with inherited gene-gene interactions. Analyses of the maternal genotypes provided evidence of a *MTHFR* C677T/*CBS* 844ins68 interaction and CTRD risk (unadjusted $P = .02$). This association is consistent with the effects of this genotype combination on folate-homocysteine biochemistry but remains to be confirmed in independent study populations.

1. Introduction

Congenital heart defects (CHDs) are a heterogeneous group of malformations with a birth prevalence that approaches 1 per 100 [1]. In addition to being the most common type of structural birth defect, CHDs have a major impact on pediatric morbidity and mortality [2]. Although CHDs can occur in association with several known teratogenic (e.g., anticonvulsants and maternal pregestational diabetes) and genetic (e.g., 22q11 deletion and Alagille) syndromes [3, 4], most CHDs (approximately 80%) appear to be nonsyndromic [5] and are thought to have a complex etiology involving interactions between several factors. However, relatively little is known about the specific risk factors for non-syndromic CHDs, and there are currently no strategies for reducing the public health impact of these conditions [3].

The identification of CHD risk factors is complicated by several issues. For example, since CHDs develop during gestation, studies aimed at identifying genetic risk factors should consider the effects of both the maternal genotype and the inherited genotype (i.e., the genotype inherited by the case) [6]. Further, as CHDs are complex conditions, the identification of risk factors may require the simultaneous assessment of multiple factors (e.g., gene-gene interactions). In addition, since CHDs are a heterogeneous group of conditions, analyses may need to be restricted to subgroups of phenotypes that are likely to be relatively homogeneous [7].

The present study was undertaken to extend our studies of the relationship between conotruncal and related heart defects (CTRD), which are thought to comprise a relatively homogenous subset of CHDs, and variation within genes in the folate metabolic pathway. This pathway was selected for analysis given evidence that, similar to neural tube defects, the risk of CHDs in general, and of CTRD in particular, is influenced by maternal folate status (reviewed in [3]), as well as variation within genes that comprise the folate metabolic pathway [8–11]. As there are few other clues regarding the causes of non-syndromic CTRD, additional studies of the association between genetic variation within the folate

metabolic pathway and the risk of CTRD are strongly warranted. Moreover, confirmation of an association between CTRD and the folate metabolic pathway would suggest potential, targeted risk reduction strategies (e.g., women with high-risk genotypes could be targeted for interventions aimed at increasing folic acid supplementation). We have previously analyzed these data using a sequential (i.e., SNP-by-SNP) approach to assess associations between CTRD and both the maternal genotype and the inherited genotype. Here, we summarize additional analyses that consider potential maternal and inherited gene-gene interaction effects, as well as heterogeneity in the effect of the inherited genotype across the CTRD component phenotypes.

2. Materials and Methods

2.1. Study Subjects. Details of this family-based, case-parent triad study have been provided previously [8]. Briefly, individuals with a CTRD were ascertained through the clinical practices of the Children's Hospital of Philadelphia between 1997 and 2007. Patients of either sex and of any race/ethnicity, with a diagnosis of tetralogy of Fallot (TOF), D-transposition of the great arteries (D-TGA), double outlet right ventricle (DORV), truncus arteriosus (TA), interrupted aortic arch (IAA), conoventricular or posterior malalignment type ventricular septal defect (VSD), or an isolated aortic arch anomaly (AAA) were eligible to be cases in this study. Medical records were reviewed to confirm the cardiac diagnosis for each case. Potential subjects who had a recognized syndrome, including the 22q11 deletion syndrome, were excluded so as to reduce etiologic heterogeneity among the cases.

Blood samples were collected from cases prior to a blood transfusion at the time of surgical or other interventions. Blood, buccal, or saliva samples were collected from each participating parent, however, participation of both parents was not required [12].

2.2. Laboratory Methods. DNA, regardless of sample type, was extracted using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's protocol. Ten polymorphisms from nine genes in the folate metabolic pathway, including eight single nucleotide polymorphisms: *BHMT* G742A (rs3733890), *MCP1* A251G (rs1024611), *MTHFR* C677T (rs1801133), *MTHFR* A1298C (rs1801131), *MTR* A2756G (rs1805087), *MTRR* A66G (rs18013940), *SHMT* C1420T (rs1979277), *TCN2* C777G (rs1801198) and two insertion/deletion alleles: *CBS* 844ins68 and *DHFR* intron 1 19-base pair deletion, were genotyped as previously described [8]. Each of these variants has been shown to influence the function of the resulting gene product [13–22]. Genotyping of single nucleotide polymorphisms was conducted in the High-Throughput Genotyping Core Laboratory at the Molecular Diagnosis and Genotyping Facility at the University of Pennsylvania, using the ABI 7900HT Sequence Detection System (Applied Biosystems, Foster City, California). The insertion/deletion polymorphisms were genotyped using published polymerase chain reaction-based assays [8, 23] and visualized on agarose gels.

2.3. Statistical Methods. All statistical analyses included a single case per family (e.g., in a family with two affected offspring, one child was randomly selected to serve as the case and the other child was not included in the study). None of the cases were known to be related. Case and parental characteristics were summarized using counts and proportions. For each analyzed genetic variant, the proportion of samples for which a genotype could not be assigned, the proportion of samples that yielded discrepant results on repeated genotypes, and the proportion of triads that had genotype combinations that were incompatible with Mendelian inheritance were determined. For each sample, the number of genotyping failures (i.e., genotypes that could not be assigned or were discrepant across repeated genotypes) was determined. These analyses were performed using SAS version 9.1 (SAS Institute Inc).

Multifactor dimensionality reduction-phenomics (MDR-P) [24] was used to assess the association between inherited gene-gene interactions and CTRD and to determine whether the association was influenced by heterogeneity across the seven specific CTRD component phenotypes included in the case definition [24]. MDR-P uses a permutation method to estimate *P*-values that are adjusted for multiple testing. All one-, two-, three-, and four-locus models derived from the ten genotyped folate metabolic genetic variants were assessed. These analyses were implemented with the computer program MDR-Phenomics [24], using data from triads with complete genotype data for all variants included in a given model.

Given that MDR-P uses information on the transmission of alleles from parents to an affected offspring to assess associations, this method cannot be used to assess maternal gene-gene interactions in case-parent triad data. Consequently, a case-only approach was used to assess maternal gene-gene interactions [25]. Specifically, for case mothers, all pairwise gene-gene combinations were assessed (e.g., *BHMT* G742A/*MCP1* A251G, *BHMT* G742A/*MTHFR* C677T). Chi-square tests were used to obtain unadjusted *P*-values. All case-only analyses were restricted to data from white mothers due to the potential for population stratification bias [26]. These analyses were performed using SAS version 9.1 (SAS Institute Inc).

Maternal gene-gene combinations that were associated with CTRD in the case-only analyses (i.e., unadjusted $P < .05$) were investigated using log-linear models for joint effects in order to obtain effect estimates [27]. To test the no-interaction null hypothesis, we calculated a 2-degree-of-freedom likelihood ratio test (LRT) statistic as twice the difference of the log likelihoods for the log-linear model that included two parameters indexing the *inherited* genotype for SNP1, two parameters indexing the *maternal* genotype for SNP1, and two interaction terms representing the product of maternal SNP1-SNP2 pairwise genotypes and a reduced model that excluded the interaction terms. These analyses were run using LEM [28], a program for log-linear

Table 1: Characteristics of study cases and parents, Children's Hospital of Philadelphia, 1997–2007.

Characteristic	Total (%) (n = 727)
Offspring sex	
Male	430 (59.2)
Female	297 (40.8)
Race/ethnicity (parental mating pairs)	
White	525 (72.2)
Black	74 (10.2)
Hispanic	22 (3.0)
Asian	20 (2.8)
Mixed	86 (11.8)
Primary diagnosis	
Tetralogy of Fallot (TOF)	279 (38.4)
D-transposition of the great artery (D-TGA)	152 (20.9)
Ventricular septal defect (VSD)*	146 (20.1)
Double outlet right ventricle (DORV)	72 (9.9)
Isolated aortic arch anomaly (AAA)	38 (5.2)
Truncus arteriosus (TA)	21 (2.9)
Interrupted aortic arch (IAA)	19 (2.6)

*Conoventricular or posterior malalignment type ventricular septal defect; coarctation of the aorta was present in 16 of the case individuals with a VSD.

analysis with missing data that allows information from triads that have not been completely genotyped (e.g., father not available) to be included in the analysis for any given variant [12]. To reduce concerns regarding possible mating stratification bias [26, 29], only data from triads in which both parents were reported to be white were used in these analyses. Given the exploratory nature of these analyses, both unadjusted and adjusted (i.e., Bonferroni corrected) P-values are presented.

3. Results

Details of the study sample have been provided elsewhere [8]. Briefly, there were 727 case-parent triads in which the case individual had a CTRD (Table 1). The most common diagnoses among the cases were tetralogy of Fallot (38.4 percent), D-transposition of the great arteries (20.9 percent), and ventricular septal defect (20.1 percent). There was a predominance of males among the cases (59.2 percent), and the majority of parents were reported to be white (72.2 percent).

DNA samples were available for 1991 members of the study triads (i.e., 537 complete triads and 190 case-parent pairs). Genotype call rates for these 1991 samples ranged from 96% to 98% for each variant; the proportion of samples that provided discrepant results on repeat genotypes ranged from 0% to 0.8%; and the proportion of triads with genotype combinations that were incompatible with Mendelian inheritance ranged from 0.7% to 2.5% (n = 5–19 families) per variant. On the basis of these results, all of the genotypes were considered to be of sufficiently high quality to include in the subsequent statistical analyses. However, all genotype data from families that included at least one genotype combination that was incompatible with Mendelian inheritance were omitted from all analyses (n = 225 samples from 75 triads with a Mendelian inconsistency for one or more variant). In addition, all genotype data from individual samples that failed (i.e., no genotype called) or provided discrepant results on repeat genotyping for four or more of the genotyped variants were omitted from all analyses (n = 55 samples). After the above-mentioned exclusions, 652 families (90%) were available for analysis, and the number of useable genotypes for each of the variants ranged from 1685 to 1715.

In our previous SNP-by-SNP analyses of these data [8], three of the ten folate metabolic variants were found to be associated with CTRD at P (unadjusted) < .05: *MTR* A2756G (maternal effect, P = .04), *CBS* 844ins68 (inherited effect, P = .05), and *MTHFR* A1298C (inherited effect, P = .002). However, only the association with the inherited *MTHFR* A1298C genotype remained significant when the false discovery rate was controlled at 0.05 (unadjusted P = .0021 < .0025).

MDR-P was used to evaluate inherited gene-gene interactions and heterogeneity across the seven CTRD component phenotypes. Using this approach, the only model achieving significance was the one-locus model for the *MTHFR* A1298C variant (adjusted P = .02). There was no evidence of heterogeneity in the association of this variant across the seven CTRD component phenotypes, and no other one-, two-, three-, or four-locus model had an adjusted P value < .05 (Table 2).

The case-only approach was used to assess the association of CTRD with maternal gene-gene interactions (Table 3). Using this approach, two maternal gene-gene combinations were associated with CTRD with unadjusted P values less than .05: *MTHFR* C677T/*CBS* 844ins68 (unadjusted P = .01) and *MTHFR* A1298C/*CBS* 844ins68 (unadjusted P = .02). Based on the data presented in Table 4, the *MTHFR* 677 TT genotype is over-represented and the *MTHFR* 1298 CC genotype is under-represented among case-mothers with the *CBS* NN genotype. As these two *MTHFR* variants are in strong linkage disequilibrium [8, 30, 31], and given prior evidence that homocysteine levels are influenced by a *MTHFR* C677T/*CBS* 844ins68 interaction [32], only the *MTHFR* C677T/*CBS* interaction was evaluated using log-linear analyses.

In the log-linear analyses, the maternal *CBS* 844ins68 IN and II genotypes were combined, due to the small number of II genotypes (n = 4), and the two resulting categories (NN versus IN + II) were used to stratify the data. An unrestricted model, which provides effect estimates for heterozygotes and for rare homozygotes, was fitted to the maternal *MTHFR* C677T genotype data. For these analyses, data from mothers who were genotyped for the *MTHFR* C677T but not the *CBS* 844ins68 variant were excluded (n = 2). Mothers who were *CBS* NN and *MTHFR* 677 TT had a 1.85-fold (95 percent confidence interval: 1.13, 3.02) higher risk of having a child with a CTRD as compared to those who were *CBS* NN and *MTHFR* 677 CC. This association was not seen in mothers with the *CBS* IN or II genotypes (Table 5). The unadjusted

Table 2: MDR-P results (adjusted *P* values) for all 2-locus models of the inherited genotype and CTRD, Children's Hospital of Philadelphia, 1997–2007.

	BHMT G742A	*CBS* 844ins68	*DHFR* 19-bp del	*MCP1* A251G	*MTHFR* C677T	*MTHFR* A1298C	*MTR* A2756G	*MTRR* A66G	*SHMT* C1420T	*TCN2* C777G
BHMT G742A		0.75	0.27	0.69	0.43	0.18	0.50	0.68	0.92	0.57
CBS 844ins68			0.40	0.38	0.59	0.09	0.48	0.92	0.45	0.63
DHFR 19-bp del				0.92	0.60	0.72	0.82	0.28	0.26	0.86
MCP1 A251G					0.58	0.83	0.60	0.53	0.47	0.67
MTHFR C677T						N/A*	0.30	0.60	0.43	0.10
MTHFR A1298C							0.13	0.23	0.13	0.32
MTR A2756G								0.57	0.13	0.74
MTRR A66G									0.88	0.18
SHMT C1420T										0.93

*This interaction was not assessed as the two *MTHFR* variants are in strong linkage disequilibrium.

Table 3: Case-only results (unadjusted *P* values) for all pairwise combinations of maternal genotypes and CTRD, Children's Hospital of Philadelphia, 1997–2007.

	BHMT G742A	*CBS* 844ins68	*DHFR* 19-bp del	*MCP1* A251G	*MTHFR* C677T	*MTHFR* A1298C	*MTR* A2756G	*MTRR* A66G	*SHMT* C1420T	*TCN2* C777G
BHMT G742A		0.95	0.64	0.71	0.15	0.56	0.92	0.06	0.38	0.92
CBS 844ins68			0.28	0.98	**0.01**	**0.02**	0.94	0.65	0.27	0.36
DHFR 19-bp del				0.95	0.41	0.59	0.14	0.81	0.14	0.17
MCP1 A251G					0.71	0.65	0.64	0.75	0.83	0.19
MTHFR C677T						N/A*	0.97	0.41	0.57	0.58
MTHFR A1298C							0.57	0.14	0.48	0.74
MTR A2756G								0.42	0.57	0.92
MTRR A66G									0.92	0.28
SHMT C1420T										0.43

*This interaction was not assessed as the two *MTHFR* variants are in strong linkage disequilibrium.

Table 4: Maternal *MTHFR* and *CBS* 844ins68 genotype combinations, Children's Hospital of Philadelphia, 1997–2007.

	CBS 844ins68			
	II	IN	NN	*P* value
MTHFR genotypes				
C677T				
CC (%)	4 (2.5)	19 (11.8)	138 (85.7)	
CT (%)	0	31 (16.0)	163 (84.0)	
TT (%)	0	2 (3.6)	54 (96.4)	.01
A1298C				
AA (%)	1 (0.5)	20 (10.2)	175 (89.3)	
AC (%)	1 (0.5)	27 (14.9)	153 (84.5)	
CC (%)	2 (6.1)	5 (15.2)	26 (78.9)	.02

P-value for the LRT of heterogeneity of the effect of the maternal *MTHFR* C677T genotype across categories defined by the maternal *CBS* 844ins68 insertion genotype was 0.02, but this association was not significant at the 0.05 level, after applying the Bonferroni correction for the evaluation of all 44 maternal gene-gene combinations ($P = .02 > .001$).

Table 5: Relative risk of CTRD for maternal *MTHFR* C677T/*CBS* 844ins68 genotype combinations estimated by log-linear regression, Children's Hospital of Philadelphia, 1997–2007.

	CBS 844ins68				
	II/IN		NN		
	RR	(95% CI)	RR	(95% CI)	*P* for interaction*
MTHFR C677T					
CC	1		1		
CT	1.26	(0.59, 2.69)	1.10	(0.79, 1.53)	
TT	0.57	(0.10, 3.45)	1.85	(1.13, 3.02)	.02

*Based on the likelihood ratio test of no interaction.

4. Discussion

We have previously reported that the inherited *MTHFR* A1298C genotype is associated with the risk of CTRD, and others have observed a similar association in CHD samples including, but not limited to CTRD [8, 9]. Our results using MDR-P are consistent with these previous findings and provide evidence that this association is similar

across the seven component CTRD phenotypes (TOF, D-TGA, VSD, DORV, AAA, TA, and IAA). However, based on these analyses, there is no evidence that the inherited *MTHFR* A1298C genotype influences the risk of CTRD via interactions with the other measured genotypes, or that other measured folate metabolic genotype combinations influence CTRD risk.

The SNP-by-SNP analyses of these data provided little evidence that the risk of CTRD is influenced by maternal genotype for any of the measured variants [8]. However, analyses of pairwise maternal gene-gene combinations suggested that a maternal *MTHFR* C677T/*CBS* 844ins68 interaction is associated with the risk of CTRD in offspring. Specifically, based on our analyses of these data, women with the *MTHFR* TT genotype appear to be at increased risk of having a child with CTRD, relative to women with CC genotype, only if they also carry the *CBS* NN genotype. Although this association did not achieve statistical significance after correction for multiple testing, it is in line with studies showing that the high homocysteine, low folate phenotype commonly observed in individuals with the *MTHFR* 677 TT genotype is present only among individuals with the *CBS* 844ins68 NN genotype [32–34]. Further, there is some evidence that interactions between these two variants may influence the risk of neural tube defects [35–38].

This study had several strengths, including a large sample size and consideration of the joint effects of genetic variants within the folate metabolic pathway. In addition, the effects of both the maternal and inherited genotypes were considered, as was heterogeneity of the effects of the inherited genotype across the component CTRD phenotypes. It is of note that in this large sample, the association between CTRD risk and the inherited *MTHFR* A1289C genotype did not appear to differ across the various CTRD phenotypes, which provides support for the inclusion of the full range of CTRD phenotypes in studies aimed at identifying CTRD risk factors.

As with all studies, this study also had limitations. Although this is the largest and most comprehensive study of CTRD and genes in the folate metabolic pathway conducted to date, it included only ten variants in nine folate metabolic genes. Further, heterogeneity within the case group may have influenced the observed associations, although results from MDR-P provide some evidence that the component CTRD phenotypes can be combined for analysis. In addition, the power to detect weak to moderate gene-gene interaction effects was low, and this limitation was further compounded by the relatively large number of interactions that were evaluated. Hence, although the observed association between CTRD and the maternal *MTHFR* C677T/*CBS* 844ins68 interaction is consistent with the biochemical consequences of this gene-gene combination, it is based on small numbers and may represent a false-positive finding.

5. Conclusions

The results of our study are consistent with the previous studies in this and other populations that indicate an association between the inherited *MTHFR* A1298C genotype and CHDs. In addition, our results suggest that this association is similar for each of the CTRD component phenotypes and, therefore, provides some support for pooling data from the component phenotypes in analyses aimed at identifying CTRD risk factors. The results of these analyses also indicate that a maternal *MTHFR* C677T/*CBS* 844ins68 interaction may be associated with the risk of CTRD in offspring. However, this finding requires confirmation in independent study samples. Hence, larger studies, which include additional folate metabolic pathway genes and a more extensive set of SNPs, are needed to more fully elucidate the role of folate in CTRD.

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Review Article

Maternal-Zygotic Epistasis and the Evolution of Genetic Diseases

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Many birth defects and genetic diseases are expressed in individuals that do not carry the disease causing alleles. Genetic diseases observed in offspring can be caused by gene expression in mothers and by interactions between gene expression in mothers and offspring. It is not clear whether the underlying pattern of gene expression (maternal versus offspring) affects the incidence of genetic disease. Here we develop a 2-locus population genetic model with epistatic interactions between a maternal gene and a zygotic gene to address this question. We show that maternal effect genes that affect disease susceptibility in offspring persist longer and at higher frequencies in a population than offspring genes with the same effects. We find that specific forms of maternal-zygotic epistasis can maintain disease causing alleles at high frequencies over a range of plausible values. Our findings suggest that the strength and form of epistasis and the underlying pattern of gene expression may greatly influence the prevalence of human genetic diseases.

1. Introduction

Why are some human genetic diseases so common? We expect natural selection to favour the alleles of genes that confer health and hinder alleles of genes that confer disease. Yet, there are a glut of genetically based human diseases, behavioural syndromes, and birth defects that occur at relatively high frequencies [1]. The classic explanation is that diseases are maintained in balance between mutation, which creates the disease, and purifying selection, which removes it [2]. Though this theory is roughly supported by diseases with simple genetic inheritance, it is not designed to apply for diseases with complex patterns of inheritance [3, 4].

Most diseases follow a complex pattern of inheritance [4–6]. One form of complex inheritance occurs when the genetic makeup of mothers influences the disease status of their offspring. This can either happen through maternal genetic effects, whereby the genotype of mothers influences trait expression in offspring, or through maternal-zygotic epistasis, whereby the phenotype of an offspring is dependent on the interaction between genes in the maternal genome and genes in the offspring genome [7, 8]. It can be viewed as kind of genotype x environment interaction, in which the maternal genome provides the environment for the offspring genotype. However, unlike standard discussions of genotype x environment interaction where the environment is fixed, the maternal environment is genetic and can evolve. The most detailed evidence for this type of inheritance comes from work in plant and animal systems [8–15], but there is also direct evidence from humans.

We do not know the full extent to which maternal genetic effects and maternal-zygotic epistasis contribute to human diseases. Our ability to detect complex inheritance is hampered by a range of factors, particularly sampling limitations, population structure, and environmental influences on disease [16–21]. Our ability to detect maternal genetic sources of inheritance is further hampered by the multigenerational complexity of the data sets required to test for it [8, 22–24]. Despite these limitations, there is emerging evidence from association studies that maternal genetic effects and maternal-zygotic epistasis might be common mechanisms of inheritance of human diseases and birth defects [16, 25–31].

The mechanism of inheritance is important to understand because it may affect the prevalence of disease. For simple genetic diseases, prevalence is determined by the mutation-selection balance [2]. However, in maternally expressed diseases half of the alleles are hidden from selection, as the genotype of fathers has no effect on offspring phenotype [32–35]. This means that purifying selection might only be half as effective at removing diseases that result from maternal gene expression. Thus, given the same mutation rate, we might expect maternal genetic diseases to have a substantially higher prevalence than zygotic genetic diseases (diseases caused by gene expression in offspring). The relaxed selective constraint on maternal genetic effects is supported by studies of population variation and diversification of maternal effect genes [34–36].

Epistasis might also contribute to the prevalence of genetic disease. Epistasis is known to confound disease gene mapping [5, 33, 37] because alleles at one locus can mask the effects of expression of alleles at another locus [38–41]. Thus, under specific forms of gene interaction, even genes that confer death in one genetic background might be maintained at higher frequencies than we would expect under traditional, mutation/selection balance, theories of disease evolution.

Previous theories have described general models of maternal genetic effects and maternal-zygotic epistasis in trait expression [7, 8, 34, 36]. However, there are many unresolved questions about their roles in genetic diseases. Do maternal genetic effects increase the incidence of genetic diseases? How do epistatic interactions between maternal and offspring genomes affect disease susceptibility? And, can models of the evolution of complex diseases assist us in identifying the genes which underlie complex diseases?

Here, we address these questions from the perspective of evolutionary population genetics. We present results from population genetic simulations of the evolution of genes affecting viability, whether owing to birth defects or adult diseases. These results are the first to demonstrate that genes that reduce fitness are more likely to be maintained in populations over time and at higher equilibrium frequencies when they are expressed in mothers than when they are expressed in offspring. The results also reveal the specific forms of maternal-fetal epistasis which increase the incidence of alleles with direct and deleterious effects on fetal survival. We show that a gene with a deleterious main effect can become fixed and mean fitness can increase because fixing a genetic background with a strong positive interaction effect may add more to mean fitness than the deleterious main effect removes. We discuss the implications of these findings for how genetic diseases evolve and for detecting genes associated with disease.

2. Materials and Methods

2.1. Statistical Definition of Diseases with Simple Genetic Inheritance. Before we can model the evolution of genetic diseases, we need to develop an appropriate decomposition of genetic effects. From the perspective of population genetics, a diseased individual has a genetic makeup that encodes lower offspring production (lower fitness) than the average individual in the population. Simple genetic diseases can be described by the additive and dominance effects that their alleles encode. The additive effect of a disease allele is half the fitness difference between the homozygote classes. The dominance effect of an allele is the deviation between the observed fitness of heterozygous individuals and the fitness midpoint between the homozygous classes. For example, the following are the genotypic values of a hypothetical gene "O", with alleles *O* and *o*, which causes lethal birth defects in all homozygous recessive offspring: OO Fitness = 1, Oo fitness = 1, oo fitness = 0. The additive and dominance values for the genotypes in this scenario are as follows: additive value for o allele = −0.5 so that the fitness of the oo genotype equals 1 + 2(−0.5); dominance value for the Oo genotype = +0.5, so that its fitness equals 1 + (+.50) + 1(−0.5). In our simulations, we equate the viability of an offspring genotype with its genotypic value. The following are the genotypic values when the hypothetical gene "O" causes lethal birth defects in 20% of homozygous recessive offspring: OO Fitness = 1, Oo fitness = 1, and oo fitness = 0.8. The additive and dominance values for this scenario are as follows: additive value = −0.1; dominance value = +0.1.

2.2. Statistical Definition of Diseases with Gene-Gene Interactions. For simple genetic diseases, we can calculate the probability that a given genotype will express the disease from the additive and dominance effects. However, when there are gene-by-gene interactions then the fitness of a given genotype is dependent on the sum of the additive and dominance values plus the effect of the interaction. There are many different classes of epistasis [8, 38–41]. We chose to focus on diploid statistical epistasis, rather than other kinds of gene interactions, because they can be estimated from genetically based epidemiological data and they describe the statistical effect of gene interactions on phenotypes in a similar manner to simple genetic effects [42]. Our goal is to illuminate the evolutionary process in terms of such statistically detectable gene effects.

Complex genetic diseases can be described by additive and dominance effects at one locus, additive and dominance effects at another locus, and all possible two-way interactions among genetic effects. In total, the additive and epistatic effects of a pair of genes (hypothetical genes "A" and "B") on phenotypes can be decomposed into eight separate orthogonal components (see Table 1). For the sake of clarity, α means additive genetics at the "A" locus, acting on genotypes AA and aa; D_A means dominance at the "A" locus, acting on the genotype Aa; β means additive genetics at the "B" locus, acting on genotypes BB and bb; D_B means dominance at the "B" locus, acting on the genotype Bb; I_{AB} means interaction between additive alleles at "A" and "B" loci, acting on AABB, aabb, aaBB, and AAbb genotypes; K_{AaB} means interaction between the Aa genotype with BB and bb genotypes; K_{ABb} means interaction between the Bb genotype with AA and aa genotypes; J_{AaBb} means interaction between Aa and Bb genotypes. For example, in Table 1, the fitness of

Table 1: The genotypic values of two zygotically expressed interacting genes. Each gene has two alleles with genetic effects decomposed into eight orthogonal components: additive (α and β, dominance (D_A and D_B), additive by additive (I_{AB}), additive by dominance (K_{ABb}), dominance by additive (K_{AaB}), and dominance by dominance (J_{AaBb}).

Genotypes	BB	Bb	bb
AA	$1+\alpha+\beta+I_{AB}$	$1+\alpha+D_B+K_{ABb}$	$1+\alpha-\beta-I_{AB}$
Aa	$1+\beta+D_A+K_{AaB}$	$1+D_A+D_B+J_{AaBb}$	$1-\beta+D_A-K_{AaB}$
aa	$1-\alpha+\beta-I_{AB}$	$1-\alpha+D_B-K_{ABb}$	$1-\alpha-\beta+I_{AB}$

Table 2: The genotypic fitness values for a pair of maternally and zygotically expressed interacting genes expressed as deviations to be added to 1. Each gene has two alleles with genetic effects decomposed into eight orthogonal components: zygotic additive (ϕ) and zygotic dominant (D_O), maternal additive (κ) and maternal dominant (D_M), zygotic additive by maternal additive (I_{OM}), zygotic dominant by maternal additive (K_{OoM}), zygotic additive by maternal dominant (K_{OMm}), and zygotic dominant by maternal dominant (J_{OoMm}).

	Offspring Genotypes		
Maternal Genotypes	OO	Oo	oo
MM	$1+\phi+\kappa+I_{OM}$	$1+\kappa+D_O+K_{OoM}$	$1-\phi+\kappa-I_{OM}$
Mm	$1+\phi+D_M+K_{OMm}$	$1+D_O+D_M+J_{OoMm}$	$1-\phi+D_M-K_{OMm}$
mm	$1+\phi-\kappa-I_{OM}$	$1-\kappa+D_O-K_{OoM}$	$1-\phi-\kappa+I_{OM}$

an individual of genotype AABB is the sum of the additive effects of the "A" and "B" loci (α and β) and their additive by additive interaction (I_{AB}).

2.3. Statistical Definition of Diseases with Maternal-Zygotic Interactions. Traditionally, epistasis has been considered for gene combinations within the same individual or genome ($g \times g$ epistasis). However, genes expressed in one individual can also interact with genes expressed in another [8, 32]. The best described form of such between-genome epistasis (often described as $G \times G$ epistasis; here we use the term maternal-zygotic epistasis to describe offspring genotype x mother genotype epistasis) is maternal-zygotic epistasis, which occurs when trait expression in offspring is determined by interactions between genes expressed in a mother and genes expressed in her offspring. Maternal genetic effects and maternal-zygotic epistasis can be described by maternally expressed additive and dominance effects at one locus, additive and dominance effects expressed in offspring, and all possible two-way interactions among genetic effects. In total, the additive and epistatic effects of a pair of hypothetical gene expressed in mothers and offspring ("M" and "O") that determine diseases in offspring can be decomposed into eight separate components (Table 2). For the sake of clarity, κ means additive genetics at the "M" locus, acting on the offspring of MM and mm mothers; D_M means dominance at the "M" locus, acting on the offspring of Mm mothers; ϕ means additive genetic effect at the "O" locus, acting on the OO and oo offspring; D_O means dominance at the "O" locus, acting on the Oo offspring. I_{MO} means interaction between additive effects of the "M" and "O" loci, acting on OO and oo offspring of MM mothers, and OO and oo offspring of mm mothers; K_{MmB} means interaction between the OO and oo offspring of Mm mothers; K_{MOo} means interaction between the Oo offspring of MM and mm mothers; J_{MmOo} means interaction between Oo offspring of Mm mothers.

Although they look similar, the fitness calculations are more complicated in transgenerational genetic models (Table 2) than physiological epistatic models (Table 1). In transgenerational genetic models the fitness of an offspring depends its mother's genotype in the previous generation. Thus, two offspring of the same genotype but with different mothers can have different viabilities. For example, in Table 2, the fitness of OOMM offspring from OOMM and OoMM mothers is the sum of the additive effects of the "O" locus (ϕ), additive effects of the "M" locus (κ) and their zygotic additive by maternal additive interaction (I_{OM}). In contrast, the fitness of OOMM offspring from OOMm and OoMm mothers is the sum of the additive effects of the "O" locus (ϕ), dominance effects of the "M" locus (D_M) and the zygotic additive by maternal dominance interaction (K_{OMm}). It is this conditioning on maternal genotype that gives maternal-zygotic gene interaction its unique evolutionary properties [7, 8].

2.4. The Model. Although the interplay between the maternal and zygotic genomes probably involves complex interactions between thousands of genes and gene products, we can begin to understand the fundamental nature of these interactions by simulating maternal-zygotic interactions under greatly simplified conditions. We consider two unlinked loci where one locus, "M," with two alleles, M and m, is expressed in mothers and influences the fitness of offspring. A second locus, "O," with two alleles, O and o, is expressed in offspring and influences the fitness of offspring. In our simulations, we also assume for simplicity standard population genetic assumptions, which include that there are diploid males and females in a population of infinite size that mate randomly and produce offspring via sexual reproduction. We assumed that there was no inbreeding depression and that fitnesses were equivalent for males and females. The model also assumes that fitness is strictly determined by the expression

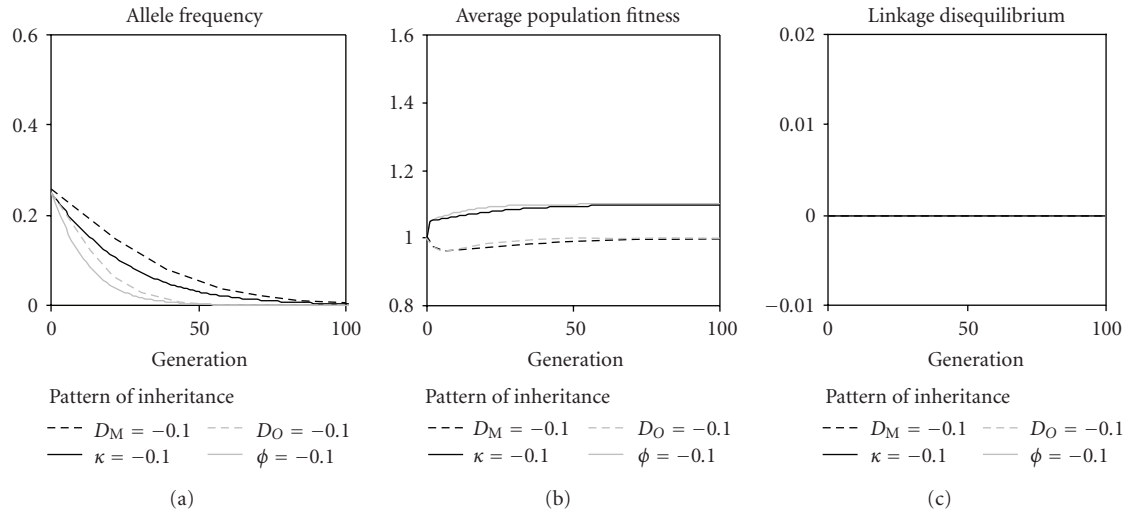

Figure 1: The evolution of genetic diseases in the absence of epistasis. The simulations track the evolution of each allele depending on its pattern of inheritance: κ, additive effect at a maternal effect locus; D_M, dominance effect at a maternal effect locus; ϕ, additive effect at a zygotic effect locus; D_O, dominance effect at a zygotic effect locus. Purifying selection is assumed to be -0.1 in all cases.

of alleles and by interactions between alleles at the "M" and "O" loci. The statistical genetic parameterization of epitasis has additional underlying genetic assumptions [42]. Fitness is assigned to offspring after they are born, but before they reproduce.

In every simulation, we set the initial starting frequency of the *m* and *o* alleles at 0.25 at two-locus Hardy-Weinberg equilibrium. Because the strength and direction of epistatic effects depends on allele frequency [37, 43] restricting the analysis to an initial allele frequency to 0.25 for both alleles limits general inferences from our simulations; however, previous models have shown that over a large range of initial allele frequencies (between 0 and 0.5) the evolutionary dynamics of maternal-zygotic interactions are determined by the relative strengths of direct selection and epistasis [43]. Our goal was to uncover some of the dynamics of how deleterious genes are maintained in populations under reasonable values of selection and epistasis, not to characterize all dynamics across all allele frequencies, selection coefficients and epistatic interactions. We chose our initial allele frequency because at a frequency of 0.25 it is easy to visualize both increases and decreases in allele frequency.

We undertook simulations to understand the evolution of genes and gene combinations which reduce fitness, our definition of genes that encode birth defects and genetic diseases. The simulations operate by measuring how the frequency of each genotype changes over 100 generations when we assign values of fitness to the sets of the parameters described in Table 2, which are the genetic effects of and epistatic interactions between the "M" and "O" genes. For each generation we also calculated the average fitness of the population and the linkage disequilibrium between the "M" and "O" loci. Linkage disequilibrium (LD), also referred to as gametic phase disequilibrium, is the nonrandom association of alleles at different loci into gametes [44, 45]. In our simulations, a positive LD means that there are a greater proportion of MO and mo gametes in the population than Mo and mO gametes. Conversely, a negative LD indicates that there are a greater proportion of Mo and mO gametes in the population than MO and mo gametes.

We conducted a total of 56 simulations of the evolution of genetic diseases under simple and complex patterns of inheritance. The following is a list of the assigned parameters used in each simulation. Purifying selection: $\kappa = -0.1$, $\phi = -0.1$, $D_M = -0.1$, and $D_O = -0.1$, each in separate simulations (Figure 1). Maternal additive by zygotic additive epistasis with and without purifying selection: $I_{MO} = -0.4$, 0, and 0.4 with $\phi = 0$ and $\kappa = 0$; $I_{MO} = -0.4$, -0.2, 0, 0.2, and 0.4 with $\phi = -0.1$ and $\kappa = 0$; $I_{MO} = -0.4$, -0.2, 0, 0.2, and 0.4 with $\phi = 0$ and $\kappa = -0.1$ (Figure 2). Maternal dominant by zygotic additive epistasis with and without purifying selection: $K_{MmO} = -0.4$, 0, and 0.4 with $\phi = 0$ and $\kappa = 0$; $K_{MmO} = -0.4$, -0.2, 0, 0.2, and 0.4 with $\phi = -0.1$ and $\kappa = 0$; $K_{MmO} = -0.4$, -0.2, 0, 0.2, and 0.4 with $\phi = 0$ and $\kappa = -0.1$ (Figure 3). Maternal additive by zygotic dominant epistasis with and without purifying selection: $K_{MOo} = -0.4$, 0, and 0.4 with $\phi = 0$ and $\kappa = 0$; $K_{MOo} = -0.4$, -0.2, 0, 0.2, and 0.4 with $\phi = -0.1$ and $\kappa = 0$; $K_{MOo} = -0.4$, -0.2, 0, 0.2, and 0.4 with $\phi = 0$ and $\kappa = -0.1$ (Figure 4). Maternal dominant by zygotic dominant epistasis with and without purifying selection: $J_{MmOo} = -0.4$, 0, and 0.4 with $\phi = 0$ and $\kappa = 0$; $J_{MmOo} = -0.4$, -0.2, 0, 0.2, and 0.4 with $\phi = -0.1$ and $\kappa = 0$; $J_{MmOo} = -0.4$, -0.2, 0, 0.2, and 0.4 with $\phi = 0$ and $\kappa = -0.1$ (Figure 5). In each case parameter values not mentioned were set at zero.

3. Results

3.1. Do Maternal Genetic Effects Increase the Incidence of Genetic Diseases? We found, as we would expect, that genes that encode alleles which reduce fitness are selectively disadvantageous. We found that additive deleterious alleles are

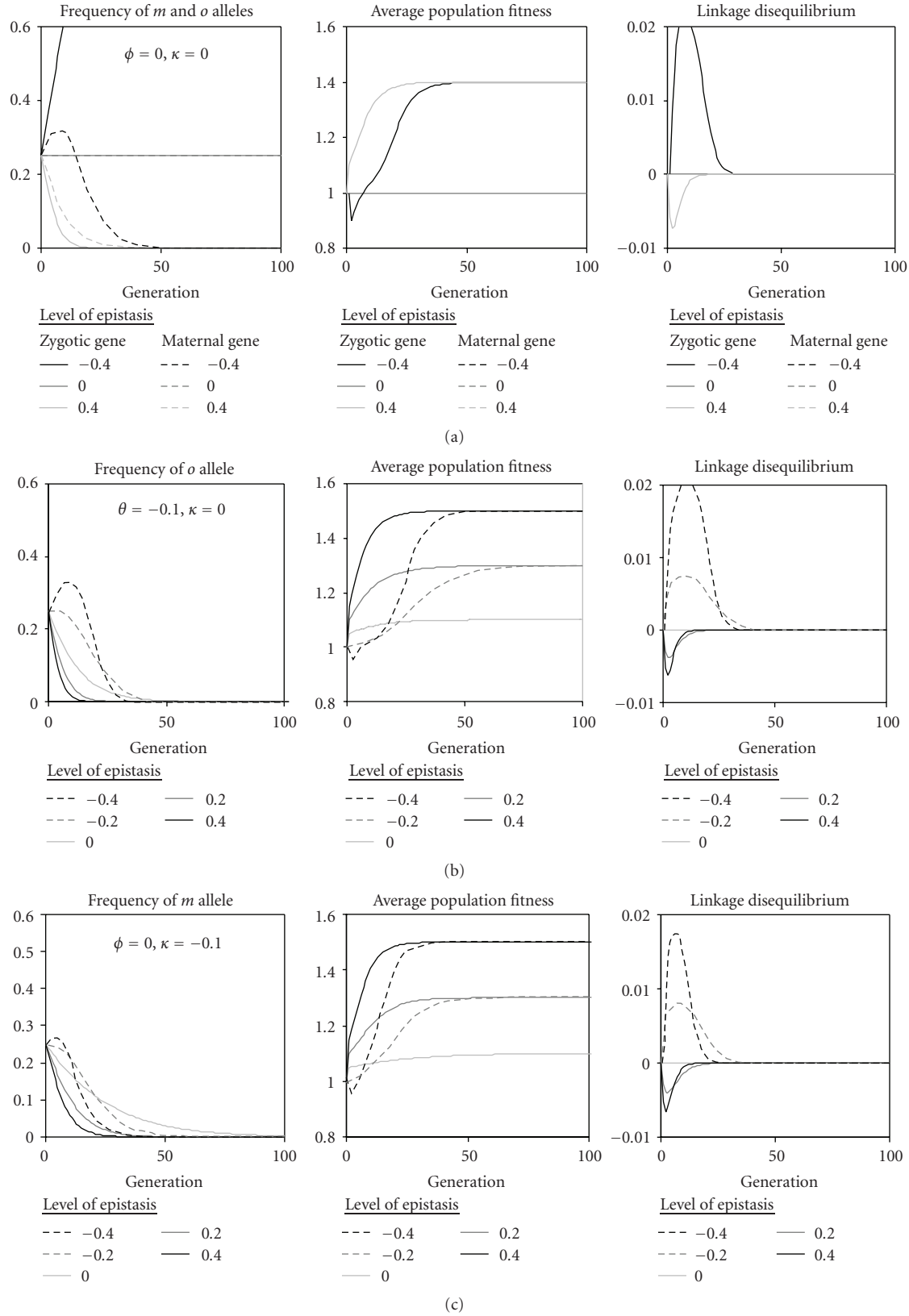

FIGURE 2: The evolution of genetic diseases under I_{MO}, maternal additive by zygotic additive epistasis. (a) Simulations which track the evolution of maternally expressed alleles and zygotically expressed alleles under different levels of epistasis without direct purifying selection. (b) Simulations which track the evolution of zygotically expressed alleles under different levels of epistasis with direct purifying selection on the zygotically expressed allele. (c) Simulations which track the evolution of maternally expressed alleles under different levels of epistasis with direct purifying selection on the maternally expressed allele.

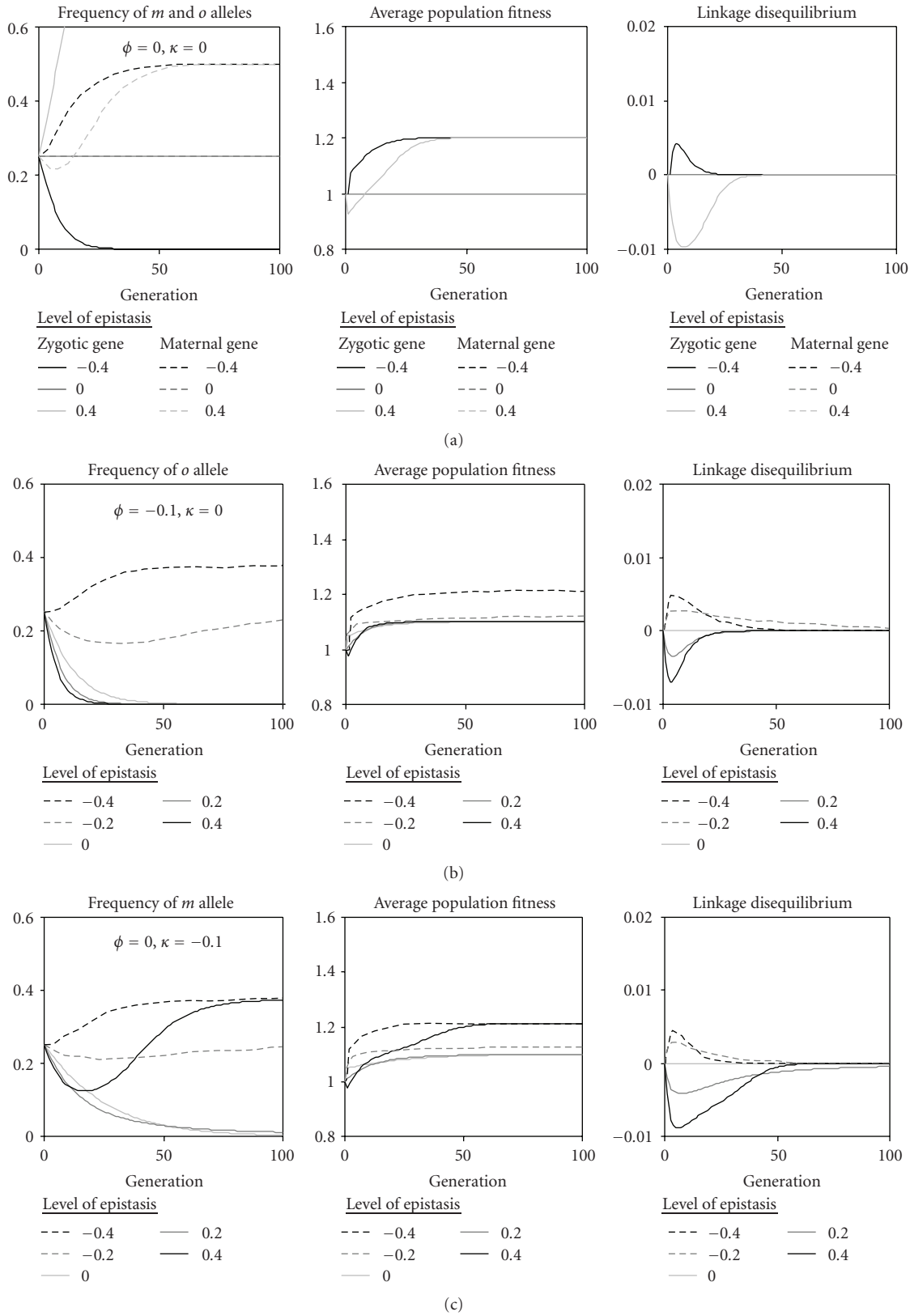

FIGURE 3: The evolution of genetic diseases under K_{OoM}, maternal additive by zygotic dominant epistasis. (a) Simulations which track the evolution of maternally expressed alleles and zygotically expressed alleles under different levels of epistasis without direct purifying selection. (b) Simulations which track the evolution of zygotically expressed alleles under different levels of epistasis with direct purifying selection on the zygotically expressed allele. (c) Simulations which track the evolution of maternally expressed alleles under different levels of epistasis with direct purifying selection on the maternally expressed allele.

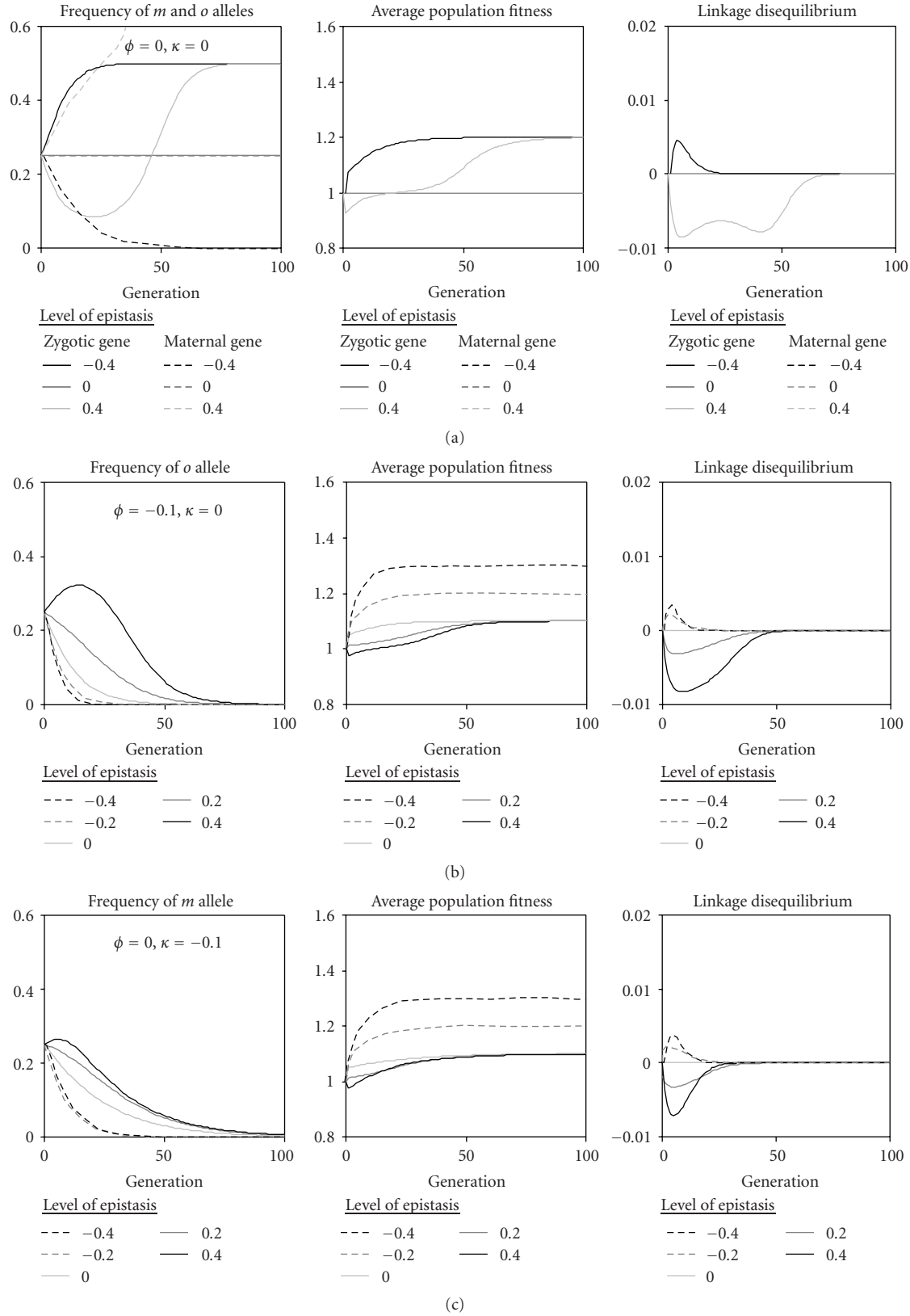

FIGURE 4: The evolution of genetic diseases under K_{OMm}, maternal dominant by zygotic additive epistasis. (a) Simulations which track the evolution of maternally expressed alleles and zygotically expressed alleles under different levels of epistasis without direct purifying selection. (b) Simulations which track the evolution of zygotically expressed alleles under different levels of epistasis with direct purifying selection on the zygotically expressed allele. (c) Simulations which track the evolution of maternally expressed alleles under different levels of epistasis with direct purifying selection on the maternally expressed allele.

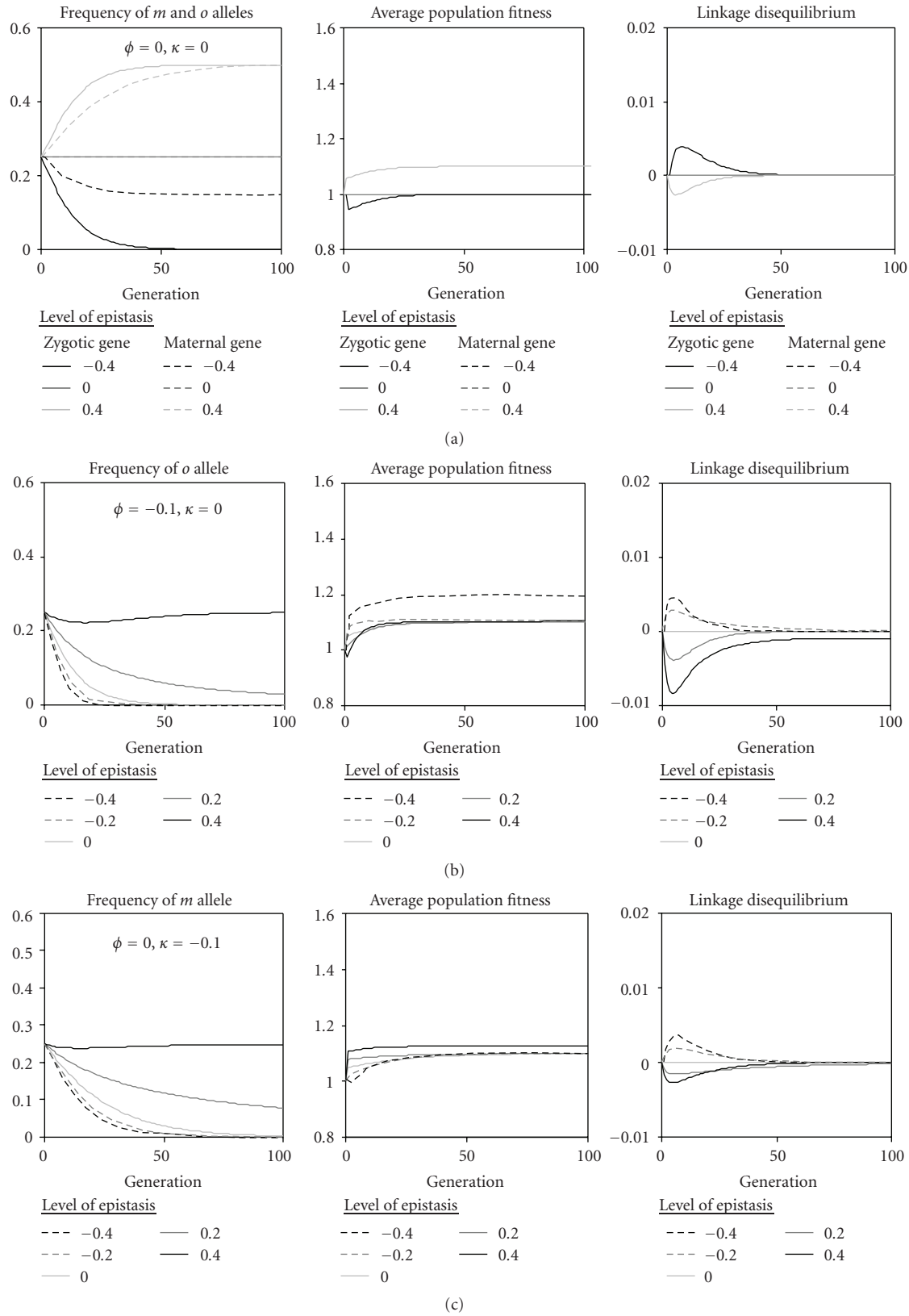

Figure 5: The evolution of genetic diseases under J_{OoMm}, maternal dominant by zygotic dominant epistasis. (a) Simulations which track the evolution of maternally expressed alleles and zygotically expressed alleles under different levels of epistasis without direct purifying selection. (b) Simulations which track the evolution of zygotically expressed alleles under different levels of epistasis with direct purifying selection on the zygotically expressed allele. (c) Simulations which track the evolution of maternally expressed alleles under different levels of epistasis with direct purifying selection on the maternally expressed allele.

lost more quickly than alleles with some level of dominance. Dominance, in effect, hides copies of the deleterious allele away from some of the action of purifying natural selection and retards the overall evolutionary process. Complete dominance hides alleles more effectively than the partial dominance we illustrate here. By comparing the maternal and zygotic patterns of gene expression, we found that maternally expressed alleles are always lost more slowly than offspring alleles with the same additive and dominance effects (Figure 1). Maternal gene expression, like dominance, effectively hides copies of the deleterious alleles in fathers, where they are not expressed and hence are hidden from the action of purifying natural selection. Note that average population fitness smoothly increases in all cases as expected when deleterious genotypes are removed by selection and their place is taken by genotypes of higher fitness. There is no LD because we are examining selection against single alleles. These results indicate that genetic diseases caused by gene expression in mothers will have a higher incidence than diseases exclusively caused by gene expression in offspring.

We see the same basic findings when purifying selection acts in conjunction with maternal-zygotic epistasis. Note that, in all cases, purifying selection acting on maternal effect genes is slower than it is on similarly acting zygotic effect genes. This reinforces and extends our general finding for single genes with only additive and dominance effects to more complex genetic architectures: maternal effect disease-causing genes will be more common in populations than similar zygotically expressed genes.

Because selection on genes with only maternal effects is weakened, we note that, in some cases, the evolutionary outcome differs for maternal and zygotic effect genes. In particular, with additive-by-dominance epistasis, strong interaction outweighs the additive, deleterious effect and produces permanent polymorphism of the maternal effect gene but not for the zygotic effect gene (compare the Left Panels of Figures 3(b) and 4(b)). This is the most dramatic case where the evolutionary equilibrium frequency of a maternal effect gene ($p_M \sim 0.36$) exceeds that of a zygotic gene ($p_O \sim 0.0$) of comparable effect.

3.2. How Do Epistatic Interactions between Maternal and Offspring Genomes Affect Disease Susceptibility? Our results show that epistasis between a maternally expressed allele and a zygotically expressed allele can alter the evolution of genetic diseases. In the absence of maternal-zygotic epistasis, purifying selection rapidly removes disease genes with additive or dominant effects (Figure 1). However, in the presence of maternal-zygotic epistasis purifying selection can have a variety of different consequences for the evolution of disease-encoding alleles. It can hasten the loss of the allele (Figures 2(a) and 2(b)). It can slow the loss of the allele (Figures 3(c), 4(b), 4(c), 5(b) and 5(c)). It can increase the frequency of the allele before eventual loss (Figures 2(b), 2(c), 4(b) and 4(c)); and it can result in permanent maintenance of disease-causing alleles (Figures 3(b), 3(c), 5(b) and 5(c)).

For additive-by-additive epistasis, the *o* allele is deleterious and, on some genetic backgrounds of the "M" gene, its deleterious effect on fitness is intensified, while on others it is diminished. In order to determine the overall effect of the interaction, the fitness of the *o* allele has to be averaged over all genetic backgrounds at the "M" locus. This averaging produces some counter intuitive results with additive-by-additive epistasis. For example, when $I_{OM} = +0.4$ (I_{OM} in Table 2 and heavy solid line in (Figure 2(a) Left Panel), the fitness of the MMOO genotype is substantially greater than 1.0 when produced by MM mothers (Fitness = 1.3 [1 − 0.1 + 0.4]), and close to 1.0 when produced by Mm mothers (Fitness = 0.9 [1 − 0.1]). Because the fitness of the MMOO genotype offspring from MM mothers is high, we might expect selection to favor the spread of *M* and *O* alleles; however, the MM genotypes also have lowered fitness in the absence of the *o* allele, so it is not clear, a priori, how the frequencies of *o* and *m* will change each generation. The simulations indicate that under positive I_{OM} epistasis both the *m* and *o* alleles are quickly removed from the population because the fitness of mmOO and MMoo intermediate genotypes is low and because oo genotypes have a fitness advantage when they are produced by mm mothers. In fact, the *m* and *o* alleles are actually lost more quickly with positive I_{OM} epistasis than with no epistasis (Figure 2(a) Left Panel). As the bad gene combinations are selected against, the mean fitness of the population is increased (Figure 2(a) Top Middle Panel). During this process, Mo and mO gametes are more common than random expectation so that LD is negative (Figure 2(a) Top Right Panel). With fixation of the *O* allele, LD returns to zero.

When we change the sign of I_{OM}, MMoo, and mmOO genotypes have the highest fitness, and the MMOO and mmoo genotypes have the lowest fitness. Here selection against the *O* disease susceptibility allele is weaker than with no epistasis because the MMoo and mmOO genotypes have higher fitness which causes the *M* allele to fix, after which in the MM genetic background, the *o* allele is no longer favored. Note that changes in mean population fitness under negative I_{OM} do not increase monotonically and that the rate of change in mean population fitness shifts as genotypes are sorted by selection (Figure 2(b) Middle Panel). During this process, OM and om gametes are more common than random expectation so that LD is positive (Figure 2(b) Middle Right Panel). However, with fixation of the *o* allele, LD returns to zero. These findings show that additive-by-additive epistasis opposite in sign of an allele's direct effect on fitness will mitigate purifying selection. Intuitively, by synergistically making a bad allele worse, selection will act to remove the allele more quickly, while in the opposite case, selection will be reduced.

Negative maternal dominance by zygotic additive epistasis appears to increase selection against additive zygotically expressed disease genes (Figure 4 Left Panels). Without epistasis, the fitnesses of the "O" locus genotypes, OO, Oo, and oo are 0.9, 1, and 1.1, respectively; however, with K_{MmO} equal to −0.4 the fitnesses are 0.5 and 0.9 for OO, 1 for Oo, and 1.1 and 1.5, for oo, depending on maternal "M" locus genotype. For the "M" locus, on the most common

"O" locus genotypic background, oo, the fitnesses are 1.1 when they are derived from MM or mm mothers and 1.5 when they are derived from Mm mothers. This is the fitness pattern of heterozygote advantage at the "M" locus (i.e., marginal overdominance or balancing selection). As a result, for negative values of K_{OoM}, the zygotic effect disease gene is rapidly lost and the maternal effect gene with no additive effect on its own evolves to a frequency of 0.5 (Figure 4(a) Top Left Panel). Mean population fitness quickly increases for negative values of epistasis (Figure 4 Middle Panels). For positive values of K_{OoM} on the most common "O" locus background there is marginal under-dominance of the fitnesses of the three "M" locus genotypes, MM, Mm, and mm, 1.1, 0.7, 1.1, respectively. Though the O disease causing allele has a substantial fitness advantage in an MM background, because the *M* allele is lost over time as a result of the marginal under-dominance, the *O* allele is concurrently lost, though more slowly and with greater levels of negative LD than without epistasis (Figure 4(c) Bottom Right Panel).

With the maternal additive by zygotic additive epistasis equal to −0.4 for mm, the most common "M" locus background, the fitnesses of the three "O" locus genotypes, OO, Oo, and oo are 0.90, 1.4, and 1.1, respectively. This is the fitness pattern of balancing selection at the "O" locus. As a result, for negative values of K_{OoM}, the frequency of the deleterious *O* allele evolves to a stable intermediate frequency (Figure 5 Left Panels). Mean population fitness is not as high as in previous cases, because at the polymorphic equilibrium, recombination and segregation produce deleterious genotypes in the population every generation (Figure 5 Middle Panels). For positive values of K_{OoM}, there is marginal under-dominance of the fitnesses of the three "O" locus genotypes, OO, Oo, and oo: 0.90, 0.6, and 1.1, respectively when in the mm maternal background. As a result, the *O* allele is lost more quickly with epistasis than without epistasis. Overall, only moderate levels of LD are generated by positive and negative dominance-by-additive epistasis (Figure 5 Left Panels).

With dominance-by-dominance epistasis and J_{OoMm} equal to +0.4 (J_{OoMm} in Table 1), again there is marginal overdominance and heterozygote advantage at the "O" locus and the population achieves a stable intermediate polymorphism. Mean population fitness is lowest at this equilibrium because recombination and segregation continue to produce deleterious offspring genotypes. It is also important to note that negative LD is generated by strong positive dominance-by-dominance epistasis for nearly 200 generations although it is absent at equilibrium (Figure 5(b) Right Panel).

When only maternal-zygotic interaction determines disease risk, the variety of possible evolutionary outcomes is greater for both genes relative to all cases that we discussed above. In particular, stable polymorphism is the most common outcome with any kind of dominance epistasis (Figures 3, 4, and 5 Left Panels). At the polymorphic equilibria, no detectable LD exists in a population (Figures 3, 4, and 5 Right Panels). As a result of the polymorphism, equilibrium disease risk and incidence will be high and, owing to the absence of LD, *nonrandom associations across the population between maternal* and *zygotic alleles, that might point us toward a causal interaction, will be lacking*. This might well represent the current state of genetic risk for maternal-zygotic diseases in the US population.

3.3. Can Models of the Evolution of Complex Diseases Assist Us in Identifying the Genes Which Underlie Complex Diseases? Our simulations provide two important lessons for the characterization of complex diseases. First, maternal genetic diseases are difficult to detect using linkage disequilibrium mapping. Maternal-zygotic epistasis is known to produce half the levels of LD as traditional epistasis [8]. Our results indicate that this finding also applies to maternal genetic disease involved in maternal-zygotic interactions. By comparing the middle and lower panels of Figures 2, 3, 4, and 5, it is clear that zygotic genetic diseases generate lower levels of LD than maternal genetic diseases when they exhibit maternal-zygotic epistasis. The implication of this finding is that studies designed to identify maternal-zygotic epistasis up to a particular effect size will need substantially larger sample sizes than studies designed to identify epistasis with effects of the same magnitude.

Second, epistasis generates patterns that, without knowledge of the exact mechanism of inheritance, appear to be similar to simple genetic effects on disease. Our results illustrate cases where allele frequencies appear be selected for or against, or can be maintained by balancing selection. Without considering the fate and frequency of the interacting alleles, these patterns could be interpreted as patterns driven by genes with simple genetic effects. But, in each of these cases the pattern is influenced by the fate and frequency of interacting loci (see [33]). This finding indicates that characterizations of simple genetic diseases might need to be qualified with the possibility that gene interactions drive the disease expression. This finding also indicates that larger, more genetically detailed data sets may provide a deeper understanding of the evolution of genetic disease.

4. Discussion

All genetic diseases are affected by the same constellation of evolutionary forces: natural selection, mutation, migration, and random genetic drift; however, the complexity of the pattern of gene expression changes the way these forces work together. The evolutionary equilibrium of simple genetic diseases is thought to be primarily determined by the mutation-selection balance [2]. However, the results of our simulations show that the pattern of gene expression (maternal versus offspring) and the form and level of gene interaction can greatly affect the incidence of genetic diseases.

Diseases genes persist longer and at higher frequencies in a population when they are expressed maternally than when they are expressed zygotically. We have shown that genes which reduce fitness evolve differently under each of the four statistical forms of epistasis. We found that genes with additive main effects on increased risk of disease can be selected for and even brought to fixation by epistasis between

maternally and zygotically expressed alleles. This was particularly true when the form of epistasis involves dominance. By tracking gene frequency changes in conjunction with the mean fitness of the population we found that as the frequencies of particular genotypes change in the population, the mean population fitness can shift in nonintuitive ways. We also found that epistasis temporarily generates LD between maternal- and zygotic-effect genes. Thus, even with the simplifying assumptions of infinite population size and random mating, maternal/zygotic epistasis had a large confounding effect on the detection of single genes underlying disease etiology.

4.1. Why Maternal Effects Occur. Maternal genetic effects and maternal-zygotic epistatic interactions on offspring phenotypes arise because maternal genotype and condition guide early embryonic development [7, 12, 13, 46, 47]. For example, it is now well established that birth weight is determined by interactions between the maternal uterine genotype and offspring genotype [8, 10, 11, 26]. Mothers contribute prenatal nutrients, mRNA, and antibodies to offspring as well as postnatal effects, most prominently during lactation [10, 11, 48]. In vertebrate embryos, maternal gene expression establishes the formation of axes and induces developmental genes in the embryo [49]. In mammals, zygotic gene expression becomes the predominant controlling factor in development between the 2 and 8-cell stage, which is called the maternal-zygotic transition (MZT) [50–52].

Perhaps the best known maternal-zygotic interaction in human disease is the Rhesus (Rh) blood factor in pregnancy [27]. Maternally expressed alleles of the RHD gene negatively interact with zygotically expressed alleles of the RHAG gene. If mothers are serologically Rh−, then exposure to serologically Rh+ molecules in fetal blood can result in the formation of antibodies against the Rh+ factor, which can result in rejection of the red blood cells of the baby and subsequent anemia, brain damage and even fetal death [27]. Similarly, maternal, zygotic and maternal-zygotic interaction effects in several genes in the folate-dependent homocysteine pathway are associated with neural tube defects [25]. Maternal genetic effects are also thought to increase the risk of Down's syndrome [28].

4.2. Maternal Condition and Gene by Environment Interaction. Though we emphasize maternal-zygotic epistasis in disease etiology, it should be noted that maternal condition also influences the health of offspring. This type of effect results from genotype-by-environment interactions. In utero exposure to smoking [31] and to alcohol [30] are probably the best knows causes of fetal syndromes, but maternal age [53, 54] and in utero exposure to disease [55] may have long-term negative impacts on offspring health [29]. Interactions between the maternal environment and genotype can also affect susceptibility to disease and birth defects. For example, the strength of effect of maternal age on the incidence of obsessive-compulsive behaviours in offspring appears to depend on interactions with the dopamine D1 gene [16].

Of course, the natural word is much more complex than our simulations, although digenic interaction models should capture much of the phenotypic variation owing to epistasis. Historically, human populations have been genetically subdivided and stratified—conditions where epistasis and maternal effect would be more important [7, 21, 33]. In the last few centuries, the development of a global economy has increased migration and as a consequence has brought genotypes together that have independently evolved for thousands of years and created populations that are unlikely to be at evolutionary genetic equilibrium. Such admixture of populations also creates LD and segregation among large blocks of genes. How can we ever hope to uncover the genetic basis of disease given this historical complexity?

4.3. Implications for Identifying Disease Genes. As one can clearly see from our simulations, complex genetic disorders have a confusing pattern of inheritance and a nonintuitive evolutionary trajectory. In the last two decades, there has been increasing interest in uncovering the genetic basis of complex diseases. Though association and linkage disequilibrium studies have identified many putative disease genes, they have often been difficult to confirm in independent population samples. Some investigators have argued that this irreproducibility is largely a consequence of weak statistical power [17, 18, 56]. However, the lack of replication of the association between genes and traits is also the signature of epistasis [33]. In some populations the genetic background will facilitate detection of disease genes, while in others, the genetic background masks detection [19, 33].

These complexities do not mean that we cannot map genetic basis of complex genetic disorders. We need to use and develop methods that identify the contexts that lead to increased risk of disease. Our simulations also show that each of the four forms of epistasis affects levels of linkage disequilibrium and, at equilibrium, LD tends toward zero. This implies that linkage association mapping of disease genes may not be that useful when the disease gene involves epistasis. Single allele-disease associations might occasionally point us in the right direction, but for most complex diseases we need approaches that embrace population subdivision and epistasis. For example, Templeton's nested cladistic analysis of phenotypic associations with haplotypes [57] identifies the specific genetic backgrounds that generate the strongest signal between alleles and disease (or any other trait) in nonexperimental populations. For particular diseases it is now possible to estimate an individual's risk of aquiring disease based on a genetic profile which classifies the individual's ethnicity [58]. There are also experimental methods that are specifically designed to detect gene interactions and maternal-zygotic gene interactions [22–24, 35]. By stratifying the population and searching for genetic sources of disease in each distinct biologically relevant clade—by embracing population subdivision and epistasis—we will be more likely to determine the genetic basis of birth defects and disease [33].

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Research Article

Detection of Fetomaternal Genotype Associations in Early-Onset Disorders: Evaluation of Different Methods and Their Application to Childhood Leukemia

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Several designs and analytical approaches have been proposed to dissect offspring from maternal genetic contributions to early-onset diseases. However, lack of parental controls halts the direct verification of the assumption of mating symmetry (MS) required to assess maternally-mediated effects. In this study, we used simulations to investigate the performance of existing methods under mating asymmetry (MA) when parents of controls are missing. Our results show that the log-linear, likelihood-based framework using a case-triad/case-control hybrid design provides valid tests for maternal genetic effects even under MA. Using this approach, we examined fetomaternal associations between 29 SNPs in 12 cell-cycle genes and childhood pre-B acute lymphoblastic leukemia (ALL). We identified putative fetomaternal effects at loci *CDKN2A* rs36228834 ($P = .017$) and *CDKN2B* rs36229158 ($P = .022$) that modulate the risk of childhood ALL. These data further corroborate the importance of the mother's genotype on the susceptibility to early-onset diseases.

1. Introduction

The risk for early-onset disorders can be influenced both by the inherited genotype of the child as well as by parentally-mediated genetic effects [1]. The mother has a crucial role in early-onset disease predisposition as she provides the prenatal environment [2, 3] and can influence her offspring's risk of disease not only as a genetic donor but also through the effects of her genes acting directly on the intrauterine milieu or indirectly through fetomaternal gene-gene interactions [1, 4–6]. Given the important role the mother can play in shaping disease susceptibility in her offspring, focusing solely on the genotype of the child in association testing could, in certain instances, be misleading.

Several family-based tests have been proposed to dissect offspring and maternal genetic contributions to early-onset disorders including the case-parent designs of Wilcox et al. [1] and Weinberg et al. [3] using a log-linear framework, and of Cordell et al. [7, 8], which uses a conditional logistic regression framework. Designs using alternative family structures have also been suggested: the hybrid design based on augmenting a set of case-parent trios with a set of parents of unrelated controls [9], the "pent" design consisting of the affected child, mother, father, and maternal grandparents [2], the case-mother/control-mother dyad design [10] or the design consisting of case-parent triads supplemented by control-mother dyads [11]. However, the use of these alternative designs can be limited by the difficulty of obtaining grandparental data or sufficiently

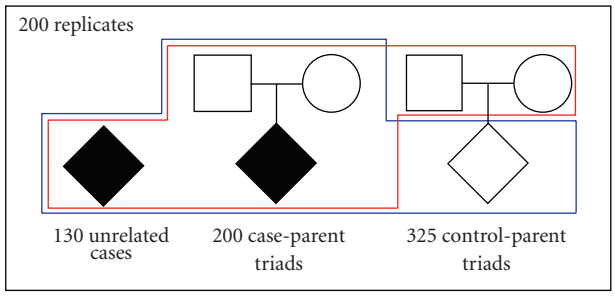

Figure 1: Cohort data used for simulations. 200 replicate datasets were generated for each model. For all of our calculations we used the same overall number of affected individuals and case-parents however the methods differed in how the case and control data were utilized in each individual test (see Materials and Methods). The same number of affected individuals ($n = 330$) and case-parents ($n = 400$) was used under all three methods. For the combined case-control and conditional logistic regression analysis (CC+CLR) and the log-linear analysis using unrelated controls rather than their parents (HD-NPC), we used the genotypes of unrelated controls ($n = 325$, in blue), whereas for the hybrid design using parents of controls in a log-linear framework (HD), we incorporated the genotypic information of the parents of these controls rather than the controls themselves ($n = 650$, in red).

large samples of parents of unaffected children, and by the increase in costs incurred for genotyping these additional sets of individuals. On the other hand, in many ongoing genetic association studies it often occurs that parents of cases and unrelated control individuals are collected in parallel, in which case designs based solely on case-triad data could lead to considerable losses in power if unrelated case-control data are disregarded. In the event that both case-parent triads as well as case-control data are ascertained, additional designs/analytical strategies are needed for fetomaternal genotype association testing.

The first aim of this study was to evaluate the adaptability of existing methods to deal with mixed data sets consisting of both case-parent triads and case-control data. We used simulations to investigate the validity and power of (1) Weinberg and Umbach's hybrid design [9] treating parents of controls as missing (HD-NPC) and (2) a classic case-control test in conjunction with Cordell et al.'s conditional logistic regression method [7] (CC+CLR), to distinguish between offspring and maternal genetic contributions to disease. Given that Cordell et al.'s approach relies solely on the use of case-parent triads, combining it with a case-control test will allow us to maximize the use of available genotype information. Finally, we compared both these approaches to a third hypothetical, ideal situation, in which genotype data from parents of controls would be available and Weinberg and Umbach's hybrid design could be used (HD) as described in [9].

However lack of parents of controls in HD-NPC and CC+CLR precludes the direct verification of the assumption of mating symmetry (MS) that is required to assess maternally-mediated effects. Mating symmetry (MS) refers to the hypothesis that for a parental genotype pair, the frequency in the population for a given mother-father genotype assignment is the same as for the reverse father-mother assignment [1, 12]. Departures from this symmetry could lead to genotype frequency differences among males and females mating in the population which, in the context of fetomaternal association testing, could lead to confounding and spurious maternal associations. As such, the HD approach, using an auxiliary sample of parents of controls to obtain direct information on mating frequencies [9], is the only method that allows for the assumption of symmetry to be directly tested and readily accommodated. It is unclear however how this method performs when parents of controls are missing and MA is present. And since parents of unaffected controls are not available in most ongoing studies, it is important to assess the robustness of these fetomaternal association tests under such circumstances. Therefore, we evaluated type I error rates and power of the three methods (HD, HD-NPC, and CC+CLR) under varying degrees of MA, and genotypic risk models involving child, mother or both child and mother jointly, in order to identify the analytical approach that is most reliable for dissecting child and maternal genetic contributions to early-onset diseases in the absence of parents of controls.

Another aim of this study was to use these methods to investigate fetomaternal associations in a real mixed dataset of childhood pre-B acute lymphoblastic leukemia (ALL) patients. ALL is a hematological malignancy resulting from chromosomal alterations and mutations that affect molecular pathways that disrupt lymphoid progenitor cell differentiation [13, 14]. There is well-established evidence for prenatal initiation of the leukemogenesis process in children [15–18]. Moreover, parental exposures to environmental carcinogens or use of medication have been identified as potential risk factors for childhood leukemia [19–23] and transplacental carcinogen exposure has been involved in the development of certain subtypes of ALL [24]. Although the risk of leukemia from environmental exposures in utero or in early childhood is likely to be influenced by genetic variation at both the level of the child and the mother, the role of maternally-mediated genetic effects in childhood leukemia susceptibility remains undefined. Here we performed a candidate gene association study using both ALL case-parent triads and unrelated controls to assess the impact of 29 SNPs from 12 cell-cycle genes in both mother and child on childhood pre-B ALL risk.

2. Materials and Methods

2.1. Methods Used to Test for Fetomaternal Genotype Associations. We compared three analytical approaches for the detection of early-onset disease associations. In the event that both case-parent triads and unrelated case-control data are available, we tested (1) a combined method in which a case-control genotypic test was carried out in conjunction with the conditional logistic regression test of Cordell et al. to detect associations at the level of the child and mother, respectively (CC+CLR); and (2) the log-linear, likelihood-based approach of Weinberg and Umbach [9]

Table 1: Forward stepwise likelihood-ratio testing procedure used to dissect child and maternal genotype associations.

Weinberg and Umbach's log-linear approach using case-triads, unrelated cases and unrelated controls (HD-NPC) or parents of controls (HD)	Genotypic case-control test combined with the conditional logistic regression approach of Cordell et al. using case-triads (CC+CLR)
Step 1.	*Step 1*.
Child genotypic effect	Child genotypic association test (CC)
Null versus CG LR Chi-square (2df)	Chi-square or Fisher's exact test (2df)
And	And
Maternal genotypic effect	Maternal genotypic effect (CLR)
Null versus MG LR Chi-square (2df)	Null versus MG LR Chi-square (2df)
Step 2. (depending on *Step 1*)	*Step 2*. (depending on *Step 1*)
Maternal effect given child effect	Maternal effect given child effect (CLR)
CG versus CG+MG LR Chi-square (2df)	CG versus CG+MG LR Chi-square (2df)
Or	Or
Child effect given maternal effect	Child effect given maternal effect (CLR)
MG versus CG+MG LR Chi-square (2df)	MG versus CG+MG LR Chi-square (2df)

Likelihood-ratio tests were performed in a forward stepwise fashion. The most significant single-step test (Child versus Null or Mother versus Null) was tested against a joint effects model in a 2 degree-of-freedom likelihood-ratio test (Child + Mother versus Child or Child + Mother versus Mother). LR Chi2 indicates likelihood-ratio chi-square test; df, degrees of freedom; GC Child genotype relative risk; GM, Mother genotype relative risk.

Table 2: The eight simulation models used for evaluation of the fetomaternal association tests.

Model	Child effect	Mother effect	MAF	ΔC
1	—	—	0.05 to 0.25	MS
2	$GC_{11} = 1; GC_{12} = 2; GC_{22} = 3$	—	0.05 to 0.25	MS
3	—	$GM_{11} = 1; GM_{12} = 2; GM_{22} = 3$	0.05 to 0.25	MS
4	$GC_{11} = 1; GC_{12} = 2; GC_{22} = 3$	$GM_{11} = 1; GM_{12} = 2; GM_{22} = 3$	0.05 to 0.25	MS
5	—	—	0.3	0 to 1
6	$GC_{11} = 1; GC_{12} = 2; GC_{22} = 3$	—	0.3	0 to 1
7	—	$GM_{11} = 1; GM_{12} = 2; GM_{22} = 3$	0.3	0 to 1
8	$GC_{11} = 1; GC_{12} = 2; GC_{22} = 3$	$GM_{11} = 1; GM_{12} = 2; GM_{22} = 3$	0.3	0 to 1

(–): indicates a null risk model where the genotype relative risks (GRRs) are $GRR_{11} = GRR_{12} = GRR_{22} = 1$. GC indicates Child genotype relative risk; GM, Mother genotype relative risk; MAF: Minor allele frequency; ΔC: mating-pair disequilibrium. MS indicates mating symmetry where $\Delta C = 0$.

using an additional set of unrelated cases and unrelated controls as proxies for parental control genotype information (HD-NPC). We also compared these two approaches to (3) one in which parents of controls are also available and therefore the hybrid design (case-parent/parents of controls) can be used through log-linear, likelihood-based analysis (HD).

It should be noted that the combined CC+CLR approach is not a modification of the conditional logistic regression approach of Cordell et al. but rather an adaptation in its use to detect fetomaternal associations. Cordell et al.'s approach relies solely on the use of case-parent triads. Since disregarding any available unrelated case-control genotype data reduces power, we used Pearson's chi-square tests or Fisher's exact tests (CC), as appropriate, and conditional logistic regression (CLR) in parallel on partially overlapping data. The former were used on all available cases to identify genotype associations at the level of the child whereas the latter was used on case-parent triads to identify maternally-contributed effects. Results from the two tests were not combined, rather if a significant association was found in the child CC test then the CLR test was used (albeit on a reduced case set) in order to dissect offspring and maternal effects. Similarly, if a significant result was found for the mother test using CLR, then CLR was further used to distinguish a main effect of the mother from a joint fetomaternal effect (see likelihood-ratio testing below).

2.2. Likelihood-Ratio Testing to Dissect Child from Maternal Genetic Effects. Given that the offspring will be enriched for the risk allele by simple Mendelian inheritance [25], it is important to discriminate between direct effects of a maternal genotype or of a child genotype from a joint fetomaternal effect. To do so, we used a forward stepwise likelihood-ratio testing procedure. In the first step, we performed two single-step tests to investigate associations at the level of the child and mother separately (Table 1). For CC+CLR, Pearson's chi-square or Fisher's exact tests were performed in R (version 2.6.2), to compare genotype distributions in cases (unrelated and triad cases) versus controls. In parallel, we used case-parent triads and logistic

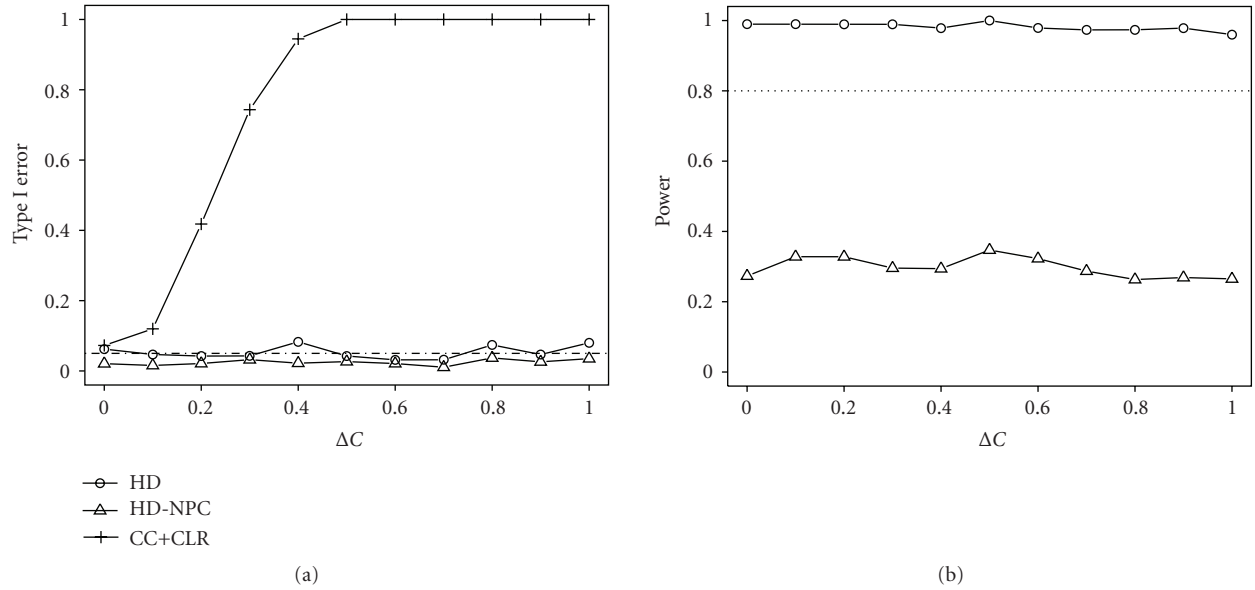

Figure 2: Type I error rates and power for the maternal association test under mating asymmetry. (a) Type I error rates are given for the Mother versus Null test as a function of departure from mating symmetry, as measured by ΔC, under the null model where $GC_{11} = GC_{12} = GC_{22} = GM_{11} = GM_{12} = GM_{22} = 1$ (Table 2, Model 5). Allele prevalence is set at $q = 0.3$. (b) Power to detect a maternal effect is shown as a function of departure from mating symmetry, as measured by ΔC, for a scenario with multiplicative effects of the mother (Table 2, Model 7). Allele prevalence is set at $q = 0.3$. MA models (9 mating-type parameters) were used for log-linear regression under the assumption of mating asymmetry. Horizontal reference lines indicate type I error rate of $\alpha = 0.05$ (a) and power = 0.8 (b). HD: hybrid design using parents of controls in a log-linear framework; HD-NPC: log-linear analysis using unrelated controls rather than their parents; CC+CLR: combined case-control and conditional logistic regression analysis.

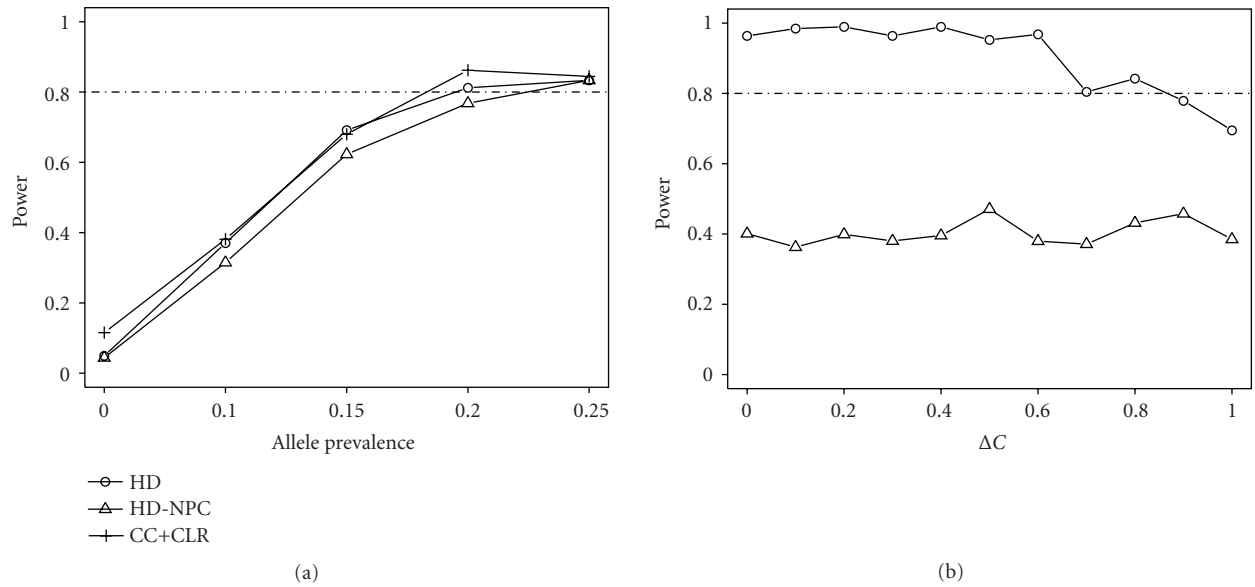

Figure 3: Power of the forward stepwise procedure to detect joint fetomaternal associations. (a) Power for the HD, HD-NPC and CC+CLR methods is shown as a function of allele prevalence for a scenario with mating symmetry and multiplicative effects of both Child and Mother (Table 2, Model 4). (b) Power for HD and HD-NPC is shown as a function of departure from mating symmetry, as measured by ΔC, for a scenario with mating asymmetry and multiplicative effects of both Child and Mother (Table 2, Model 8). Allele prevalence is set at $q = 0.3$. MA models (9 mating-type parameters) were used for log-linear regression under the assumption of mating asymmetry. Horizontal reference lines indicate power = 0.8. HD, hybrid design using parents of controls in a log-linear framework; HD-NPC: log-linear analysis using unrelated controls rather than their parents; CC+CLR: combined case-control and conditional logistic regression analysis.

Table 3: Parental genotype distributions under mating symmetry and mating asymmetry.

Mating type	Parental genotypes (Mother-Father)	Expected distribution Mating symmetry	Mating asymmetry
0	11-11	$N * \mu_0$	$N * \mu_0$
1	11-12	$N * \mu_1$	$(2 - C_1) * N * \mu_1$
	12-11	$N * \mu_1$	$C_1 * N * \mu_1$
2	11-22	$N * \mu_2$	$(2 - C_2) * N * \mu_2$
	22-11	$N * \mu_2$	$C_2 * N * \mu_2$
3	12-12	$N * \mu_3$	$N * \mu_3$
4	12-22	$N * \mu_4$	$(2 - C_4) * N * \mu_4$
	22-12	$N * \mu_4$	$C_4 * N * \mu_4$
5	22-22	$N * \mu_5$	$N * \mu_5$

Alleles for a biallelic locus are denoted 1 and 2 and the corresponding genotypes 11, 12 or 22. N indicates the number of individuals in the sample; μ_i the ith mating type probability under the assumption of random mating; C_i the mating-pair disequilibrium for the ith parental couple. C is a multiplicative factor between 0 and 2 that describes the over representation (>1), under representation (<1) or symmetry (=1) of a mate-pair combination in the corresponding ith mating type.

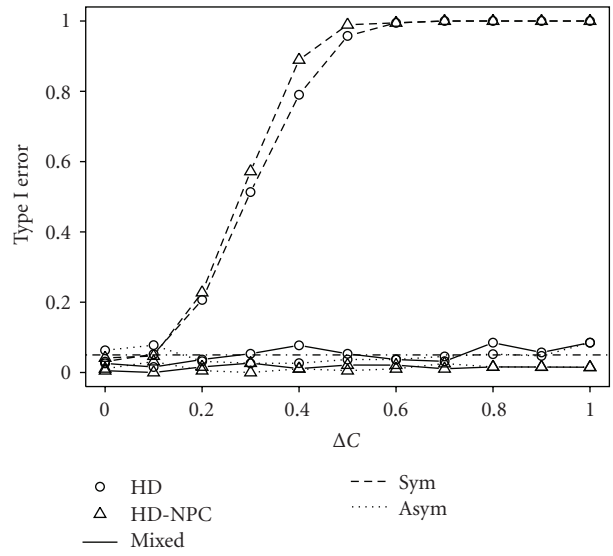

Figure 4: Type I error rate for the HD and HD-NPC approaches for the maternal association test assuming either mating symmetry or asymmetry. Type I error rates are shown as a function of departure from mating symmetry, as measured by ΔC, under the null model where $GC_{11} = GC_{12} = GC_{22} = GM_{11} = GM_{12} = GM_{22} = 1$ (Table 2, Model 5). Allele prevalence is set at $q = 0.3$. Log-linear, likelihood-based testing (Mother versus Null) was performed assuming either mating asymmetry estimating nine mating-type parameters in the likelihood-ratio tests (Asym), or mating symmetry using only six mating-type parameters in the likelihood-ratio tests (Sym), or with no a priori hypothesis, using a 3 degree-of-freedom likelihood-ratio test to first test the assumption of mating symmetry (Mating Asymmetry (9df) versus Mating Symmetry (6df)) and then using either the mating asymmetry models when asymmetry was detected or the mating symmetry models when it was not (Mixed). Horizontal reference line indicates type I error rate of $\alpha = 0.05$. HD, hybrid design using parents of controls in a log-linear framework; HD-NPC, log-linear analysis using unrelated controls rather than their parents.

regression conditioning on exchangeable parental genotypes (CEPG) [7, 8], using the DGCgenetics package for R developed by D. Clayton (available at http://www-gene.cimr.cam.ac.uk/clayton/software/DGCgenetics_1.0.tar.gz) to test for maternally-mediated associations through a two degree-of-freedom likelihood-ratio test (Mother versus Null). For HD-NPC and HD, log-linear regression analysis was performed using the LEM software (Log-linear and event history analysis with missing data using the EM algorithm) [26] and LEM script files provided by Weinberg et al. (available at http://www.niehs.nih.gov/research/resources/software/hybrid/index.cfm) [9, 26]. Two degree-of-freedom likelihood-ratio tests were performed to independently test for offspring (Child versus Null) and maternal (Mother versus Null) associations in the log-linear framework.

In the second step, if a significant effect was found in the first step (based on a Bonferroni correction of $P < .025$ for the 2 tests performed), then the most significant model was tested against a joint effects model (Child + Mother versus Child or Child + Mother versus Mother) in a two degree-of-freedom likelihood-ratio test (Table 1). For CC+CLR this meant that conditional logistic regression was used on the reduced case-triad data set to test either Child + Mother versus Child or Child + Mother versus Mother depending on the results from step one. Whereas log-linear regression analysis was performed on case-triads and unrelated cases and controls in HD-NPC and on case-triads, unrelated cases and parents of controls in HD.

2.3. Simulated Data. We simulated cohort data under several conditions to model different genotypic risk effects involving either child, mother or both child and mother (joint feto-maternal effects) (Table 2). Under each model 200 replicate datasets were simulated using the R software. Replicates that did not fit the simulated models were not included in the analysis. To imitate our childhood leukemia cohort, we simulated genotypic data for 200 case-parent triads, 130 unrelated cases and 325 unrelated control-parent triads for each replicate. For all of our calculations we used the same overall number of affected individuals however the methods differed in how the case and control data were utilized in each individual test (Figure 1). For all of our child-based tests we used the same number of affected individuals and unrelated controls (Figure 1): 330 cases (200 case-triads and 130 unrelated cases) were compared to 325 unrelated controls. However the number of cases and population-based controls used for maternal and fetomaternal association testing varied depending on the method: CC+CLR used the genotypes from 200 case-triads only; HD-NPC used the genotypes of 330 cases (200 case-triads and 130 unrelated cases) and of 325 unrelated controls; and HD incorporated the genotypic information of the parents ($n = 650$) rather than the unrelated controls themselves (Figure 1).

In HD and HD-NPC, unrelated cases and controls were used by treating their parental genotypes as missing and the

EM algorithm implemented in the LEM software was used to infer missing genotype information [26]. Unrelated cases and cases belonging to triads were considered to have similar penetrance and thus similar genotypic relative risks (GRR). We assumed Hardy-Weinberg equilibrium and the absence of population stratification in the form of admixture. We evaluated the different approaches in terms of type I error rate and power to detect associations by counting the number of replicates found to be significantly associated over the total number of replicates that fit the specified model.

2.4. Simulated Scenarios. We first assessed the behaviour of the three methods in scenarios in which MS was assumed across parents. Under MS, HD-NPC and HD were performed by forcing six mating-type variables in the log-linear model [9].

A second set of simulations was performed in which we assessed the performance of the tests assuming differences in genotype frequencies between males and females mating in the population, that is, assuming varying levels of mating asymmetry. MA was evaluated in terms of the degree of departure from the expected mate-pair probability under symmetry. We used C_i to denote the disequilibrium for the ith parental mating type. C_i is a multiplicative factor between 0 and 2 that describes the over representation ($C > 1$), under representation ($C < 1$) or symmetry ($C = 1$) of a mate-pair combination in the corresponding ith mating type. The level of departure from MS is denoted as ΔC, a numerical value ranging from −1 to 1, with $C = 1 + \Delta C$. The expected parental genotype distributions under the assumption of MS and MA are shown in Table 3. By varying the departure from mating symmetry, ΔC, we introduced varying levels of asymmetry into our simulations (Table 2). In this study we assumed a model for MA where $C_1 = C_2$ and $C_4 = 1$. Supplementary Figure 1, mentioned in Supplementary Material that is available online at doi: 10.1155/2010/369534, shows how departures from symmetry, as measured by ΔC, translate into differences in reciprocal mating types and overall genotype frequencies between males and females mating in the population. Under MA, HD-NPC and HD were performed by forcing nine mating-type variables in the log-linear model [9] and we tested for the presence of asymmetry by comparing the nine mating-type model to the six mating-type model with a three degree-of-freedom likelihood-ratio test using a cut-off of $P < .05$ to declare deviation from symmetry. A less conservative P value <.10 was also used (data not shown) and yielded similar results.

2.5. Childhood Acute Lymphoblastic Leukemia Data

2.5.1. Study Subjects. We investigated fetomaternal associations in a pre-B acute lymphoblastic leukemia (ALL) cohort. The study population has been previously described [27, 28]. Briefly, incident cases of childhood pre-B ALL ($n = 321$) were diagnosed in the Division of Hematology-Oncology of the Sainte-Justine Hospital in Montreal, Canada, between October 1985 and November 2006. Our cohort includes 189 boys and 132 girls with a median age of 4.7 years, all French-Canadian from the province of Quebec, Canada. Parental DNA was available for 203 of the probands. Healthy controls ($n = 329$) consisted of French-Canadian individuals recruited while using clinical departments other than Hematology-Oncology of the Sainte-Justine Hospital.

2.5.2. SNPs, Genotyping and Quality Control Checks. We selected 29 SNPs from 12 candidate cell-cycle genes for the analysis (Table 4). Genes were selected based on their function in regulating the G1/S cell-cycle checkpoint. Regulatory SNPs (found to lie within the proximal promoter region) were chosen based on the hypothesis that variation in gene dosage of such critical cell-cycle genes due to functional regulatory polymorphisms could influence cancer susceptibility by altering cell homeostasis [28]. For the purpose of this study using a French-Canadian cohort, we considered European-specific SNPs previously identified in [29]. DNA was isolated from buccal epithelial cells, peripheral blood or bone marrow in remission as previously described [30]. SNPs were genotyped using the Luminex xMAP/Autoplex Analyser CS1000 system (Perkin Elmer, Waltham, MA). Genetic variants were amplified using allele-specific primer extension in multiplexed assays and hybridized to Luminex MicroPlex TM –xTAG Microsperes as per Koo et al. [31]. Primer sequences, amplification conditions, and reaction conditions are available upon request. Genotypes were called using the Automatic Luminex Genotyping (ALG) software [32]. Three negative controls and three sample duplicates were used on each 96-well DNA plate. The average genotype call rate was 99.8% and rates of discordance were below 3.3%. In addition, Hardy-Weinberg equilibrium was tested using the X^2 goodness of fit test and PedCheck (Version 1.1) was used to identify genotype incompatibilities using the familial data [33]; inconsistent case-parent trios were removed from the analyses. Multiple testing corrections were performed on the single-step association tests using the Benjamini-Hochberg false discovery rate (FDR) method with a type I error rate of 10%; nominal P values are shown.

3. Results and Discussion

Although there are currently no data to document the frequency of events that lead to mating distortions in human populations, it is biologically plausible that MA might commonly occur. It is known that assortative mating (selection of a mate on the basis of phenotype leading to correlation between phenotypes of mated individuals with respect to a given trait) can lead to genotype frequency differences between males and females [34–36]. Other mechanisms leading to mating asymmetry however are unclear and their evolutionary consequences much less understood. If, for a specific marker, MA results in a departure from Hardy-Weinberg equilibrium, this genetic marker would be excluded from an association study following quality control. However MA could also arise through mating selection but with discrimination acting oppositely in each sex, or through differential individual mating success for the genotypes of each sex. Both these processes could lead to genotype

Table 4: Genes and DNA variants genotyped in the pre-B ALL association study.

Gene (Chromosme)	DNA variant	Position	MAF
CCND1 (11q13)			
	rs1944129	69,163,116	0.4876
	rs36225395	69,163,517	0.4523
CDC25a (3p21)			
	rs1903061	48,206,923	0.1028
CDKN1A (6p21)			
	rs733590	36,753,181	0.3616
	rs762624	36,753,566	0.2714
	rs2395655	36,753,674	0.3968
CDKN1B (12p13)			
	rs3759217	12,759,719	0.1159
	rs35756741	12,759,968	0.0865
	rs36228499	12,761,203	0.4342
CDKN2A (9p21)			
	rs36228834	21,965,319	0.0512
CDKN2B (9p21)			
	rs36229158	22,000,681	0.0282
	rs2069416	22,000,004	0.3742/0.0271
	rs2069418	21,999,698	0.4272
E2F1 (20q11)			
	rs3213141	31,738,041	0.2405
HDAC1 (1p35)			
	rs1741981	32,529,026	0.3302
	rs36212121	32,529,102	0.0031
	rs36212119	32,529,840	0.0846
MADH3 (15q22)			
	rs36221701	65,143,543	0.1199
	rs36222034	65,144,732	0.1111
	rs11633026	65,144,812	0.1235
MDM2 (12q15)			
	rs1144944	67,486,752	0.4954
	rs3730485	67,487,073–67,487,112	0.4052
	rs937282	67,488,064	0.483
	rs2279744	67,488,847	0.3662
RB1 (13q14)			
	rs1573601	47,774,358	0.2484
TGFB1 (19q13)			
	rs2317130	46,553,514	0.3141
	rs4803457	46,553,199	0.3937
	rs11466313	46,553,177–46,553,178	0.3096
	rs1800469	46,552,136	0.3127

DNA variant positions relative to dbSNP build 130. MAF indicates minor allele frequency and was calculated on a control cohort consisting of 329 healthy individuals of European descent.

frequency differences between sexes that would not lead to detectable deviations from Hardy-Weinberg equilibrium but that may incur important biases in fetomaternal association testing. In addition to these biological causes, low levels of MA could arise in a study sample simply due to the sampling process. Although parents of controls allow direct testing for bias due to MA in fetomaternal genotype association testing [9], these samples are difficult to collect and a method that can combine case-control and family-based data and provide a valid analytical framework for fetomaternal association testing in the presence of MA is currently not available.

3.1. Simulation Study. In this study we used simulations to investigate the ability of three fetomaternal genotype association tests: (1) the log-linear, likelihood-based method of Weinberg and Umbach [9] using a case-parent/case-control design (HD-NPC), (2) the conditional logistic regression approach of Cordell et al. [7] combined with a case-control test (CC+CLR), and (3) Weinberg and Umbach's hybrid design using log-linear regression analysis, to distinguish between offspring and maternal genetic contributions to disease in the presence of MA. It should be noted that since both the log-linear and CLR frameworks are equivalent in terms of power and type I error for the detection of maternal genotype effects [7], our conclusions regarding the CC+CLR approach would also apply to a case-control combined with the log-linear linear framework of Wilcox et al. [1] and Weinberg et al. [3] using case-parent triads. We evaluated type I error rates and power of the methods under varying degrees of MA, and genotypic risk models involving child, mother or joint effects of both child and mother (Table 2). For clarity reasons, we present the results for multiplicative genotype effect models only. However similar results were obtained under dominant and recessive models, with recessive models yielding expected decreases in power across all methods, particularly at low allele frequencies.

As expected, all three methods showed similar low type I error rates, around 5% and similar power, above 80% for the detection of child effects ($GC_{11} = 1$; $GC_{12} = 2$; $GC_{22} = 3$) under MS as well as under MA (data not shown). For a maternal main effect ($GM_{11} = 1$; $GM_{12} = 2$; $GM_{22} = 3$), type I error and power under MS were also within the acceptable ranges (data not shown). By contrast, under MA the CC+CLR method yielded unacceptably high type I error rates for the Mother test (Figure 2(a)). Although we expected that the method developed by Cordell et al. would be susceptible to the confounding incurred by MA, we found that CLR does not withstand even low levels of asymmetry ($\Delta C \sim 0.1$) so that even weak assumptions concerning population distributions of parental genotypes could lead to important bias.

The validity of the Mother tests for HD and HD-NPC were unaffected by MA, with type I error rates below the 5% threshold (Figure 2(a)). Power of the HD design was maintained at 100% and was unaffected by MA under the simulation conditions considered here, whereas power of HD-NPC considerably dropped, averaging around 30% (Figure 2(b)). When HD-NPC is used under asymmetry, genotypes for the parents of controls are inferred based on mating-type frequencies estimated from the parents of the cases and based on the assumptions that the control offspring genotypes follow Mendelian proportions in relation to their parents [9]. Hence, the maternal effect present in the case

triads is partially captured in the inference of the mating-type frequencies for the parents of controls, resulting in a loss of power to detect this maternal effect as it becomes confounded with the estimated asymmetry.

The stepwise procedure allows maternal and case effects to be distinguished by estimating maternal effects independently of offspring effects and provides a valid test for joint fetomaternal associations. Under the null model and MS, the stepwise likelihood-ratio testing procedure yielded type I error rates close to 0% (data not shown), most likely due to the over-conservative Bonferroni correction that was applied. Since the Mother and Child tests are not completely independent a permutation test procedure would provide less-conservative type I error estimates. When we modelled multiplicative effects of both offspring and maternal effects ($GC_{11} = GM_{11} = 2$ and $GC_{12} = GM_{12} = 3$), the power to detect fetomaternal associations using the forward stepwise procedure was comparable for all three methods and increased with increasing allele prevalence for each method, reaching 80% for MAFs > 0.20 (Figure 3(a)).

We then evaluated type I error rates and power for the stepwise procedure in the presence of MA. The performance of both HD and HD-NPC was unaffected by MA with type I error rates close to zero even in the presence of high levels of asymmetry (Supplementary Figure 2). The type I error rate of the CC + CLR approach was close to 5% (Supplementary Figure 2) given that both child and maternal effects had to be falsely detected in order for the replicate to be counted as a false-positive and the case-control component of the test was robust against spurious child associations. Because the CLR maternal test is not valid under even low levels of MA, we assessed the power of the other two approaches to detect fetomaternal associations for varying levels of MA (Figure 3(b)). HD performed significantly better than HD-NPC: sensitivity of the HD design averaged around 100% whereas power of HD-NPC was again significantly lower, averaging around 40%.

Based on the above findings, no method seemed to provide a net advantage under MS for these simulation conditions; nor did we observe any significant loss of power or robustness when the conditional logistic regression or the log-linear, likelihood-based approaches were used without parents of controls. Although power was significantly reduced, the log-linear, likelihood-based approach using controls rather than parents had little effect on the specificity of the association tests in the presence of MA. However, for the analyses performed on MA simulated datasets, we forced the estimation of nine mating-type parameters. In practice, no a priori assumptions regarding MS could be made. To verify the robustness of both methods for the detection of maternal (and fetomaternal) genotype effects in the presence of asymmetry, we measured type I error rates for the Mother test for scenarios in which either MA or MS models are assumed, and for a scenario in which no a priori hypothesis is made but rather MS is first evaluated in a three degree-of-freedom likelihood ratio test and the appropriate models (MA or MS) are subsequently used for association testing. These results show that if one assumes MS and this assumption is violated, type I error rates for the maternal test for both HD-NPC and HD are significantly inflated (Figure 4). However, first testing for asymmetry and then adjusting the association analyses accordingly provides accurate type I error rates for both methods. Similar results were obtained for the forward stepwise procedure (data not shown).

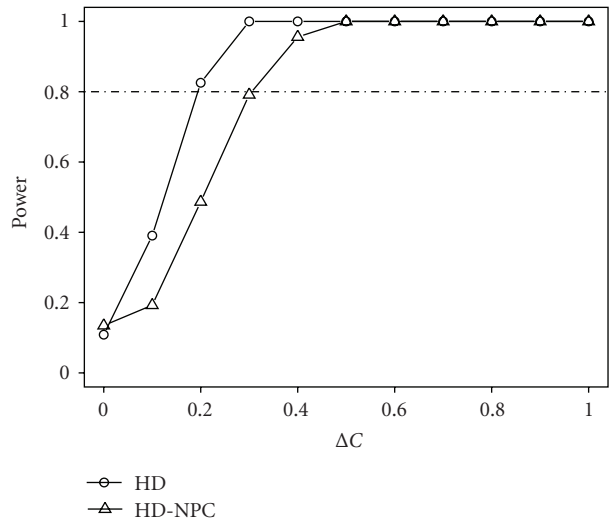

Figure 5: Power of HD and HD-NPC to detect mating asymmetry. Power is shown as a function of departure from mating symmetry, as measured by ΔC, under the null model where $GC_{11} = GC_{12} = GC_{22} = GM_{11} = GM_{12} = GM_{22} = 1$ (Table 2, Model 5). Allele prevalence is set at $q = 0.3$. Mating asymmetry was evaluated in the log-linear framework using a 3 degree-of-freedom likelihood-ratio test comparing the 9 mating-type parameter model under MA to a 6 mating-type parameter model under MS. Horizontal reference line indicates power = 0.8. HD: hybrid design using parents of controls in a log-linear framework; HD-NPC: log-linear analysis using unrelated controls rather than their parents.

The specificity of the HD-NPC test therefore relies on its ability to detect MA and then use mating-type models accounting for asymmetry to test for association. Given that there are no biological references for the amount of MA that occurs in human populations, we evaluated the capacity of the HD and HD-NPC methods to detect various levels of MA (Figure 5). Our simulation results showed that the power of HD-NPC to detect asymmetry above $\Delta C = 0.4$ was comparable to that of HD. For a risk allele frequency of $q = 0.3$, HD and HD-NPC reached 80% power at $\Delta C = 0.25$ and $\Delta C = 0.35$, respectively. Under low levels (ΔC ranging from 0 to 0.2) the sensitivity of both methods to detect asymmetry was threatened, especially for the HD-NPC approach (Figure 5). On the other hand, the lack of power of HD-NPC and HD to detect low levels of asymmetry is compensated by the fact that, without any a priori assumptions regarding mating symmetry, both methods maintained low type I error rates, at least under the simulation conditions presented here (Figure 4).

Therefore if asymmetry is not strong enough to be detected by the MA test it should not be falsely interpreted

Table 5: Distribution of *CDKN2A* rs36228834 and *CDKN2B* rs36229158 genotypes and associated risk estimates for pre-B ALL susceptibility among children.

Gene, DNA variant,	No. (%)				Log-linear regression analysis			
and genotype	ALL patients	ALL mothers	ALL fathers	Controls	Model	Genotype	Child OR (95% CI)	*P*
CDKN2A								
rs36228834					Child versus Null	TA versus TT	2.48 (1.45–4.15)	.001
TT	266 (86.6)	160 (93.0)	149 (86.6)	298 (93.7)		AA versus TT	9.87 (0.89–109.69)	
TA	39 (12.7)	12 (7.0)	22 (12.8)	19 (6.0)		TA/AA versus TT	2.56 (1.54–4.26)	<.0005
AA	2 (0.7)	0	1 (0.6)	1 (0.3)	Child + Mother versus Null	TA versus TT	3.13 (1.81–5.40)	<.0005
						AA versus TT	—	
						TA/AA versus TT	2.56 (1.54–4.26)	<.0005
CDKN2B								
rs36229158					Child versus Null	CT versus CC	1.77 (0.98–3.21)	.054
CC	277 (91.4)	164 (95.4)	155 (90.1)	302 (94.7)		TT versus CC	8.25 (0.75–91.3)	
CT	24 (7.9)	8 (4.6)	16 (9.3)	16 (5.0)		CT/TT versus CC	1.86 (1.04–3.34)	.037
TT	2 (0.7)	0	1 (0.6)	1 (0.3)	Child + Mother versus Null	CT versus CC	2.32 (1.23–4.35)	.033
						TT versus CC	—	
						CT/TT versus CC	2.44 (1.29–4.60)	.006

Percentages indicate number of individuals with a given genotype/total number of genotyped individuals. Risk estimation was performed using log-linear regression analysis as implemented in the LEM software. Child odd ratios were measured using regression models consisting of the child genotype effect only (Child versus Null) or both child and mother genotypes (Child + Mother versus Null). Mating symmetry (i.e., six mating-type parameters) was assumed at both loci. *P* values of the Wald test provided by LEM are shown for either the 2 degree-of-freedom (2 child genotype effects) or 1 degree-of-freedom (1 child genotype effect resulting from the collapsed heterozygous/homozygous rare genotypes) tests. OR indicates odds ratio; CI: confidence interval.

as a maternally-mediated effect. By contrast if a maternal effect is present and HD-NPC is used to test for mating asymmetry without parents of controls, type I error rates (of false detection of MA) are high (ranging from 0.8 to 0.9 for MAFs of 0.10 to 0.25, resp.) leading to a subsequent loss of power to detect maternal effects due to the over-parameterisation under the MA models (data not shown). Together these results show that the log-linear, likelihood-based stepwise procedure using unaffected offspring provides a valid framework to evaluate MS without leading to spurious maternal associations. And when parents of controls cannot be ascertained but an additional set of unrelated controls is available, one can safely use this approach to test for fetomaternal associations if willing to accept that certain confounded maternally-mediated effects may be missed when parental mating is asymmetric.

3.2. Fetomaternal Association Study of Childhood Acute Lymphoblastic Leukemia. Guided by our simulation results we went on to test for fetomaternal associations between 29 SNPs in the proximal promoter regions of 12 cell-cycle genes [29] and the susceptibility to childhood pre-B ALL. SNP frequencies were in agreement with those previously reported in other populations of European descent and all distributions were in Hardy-Weinberg equilibrium. Our dataset consisted of 118 pre-B ALL patients, 203 ALL case-parent triads, and 329 unrelated controls. The lack of parents of controls prevents us from excluding MA in the source population. Based on the results from our simulation study, we used the log-linear framework to perform likelihood-based testing in a stepwise fashion. For each SNP, we performed a three degree-of-freedom likelihood-ratio test for asymmetry implemented in the LEM software using a slightly less stringent *P* value <.10 to reject symmetry in order to reduce false-positives in the tests for maternal effects. Under this threshold, we identified MA at variants rs1144944 ($P = .086$) and rs3730485 ($P = .095$) of the *MDM2* gene, as well as at *CDKN2B* variant rs2069416 ($P = .076$); we did not detect asymmetry at any of the remaining loci tested (*P* values >.10; data not shown). Consequently, MA models (nine mating-type parameters) were used to test for association at these three SNPs whereas MS models (six mating-type parameters) were used for the remaining 26 SNPs (see Figure 6, for the Child and Mother single-step test results and Supplementary Table 1 for complete likelihood-ratio chi-square test results). Nominally significant genotype associations at the level of the child were identified for *CDKN2A* rs36228834 (Child versus Null; $P = .0007$), *CDKN1B* rs35756741 (Child versus Null; $P = .0235$) and *CDKN2B* rs2069416 (Child versus Null; P= 0.0063); however only *CDKN2A* rs36228834 and *CDKN2B* rs2069416 remained significant after multiple testing corrections (Supplementary Table 1). None of the other 26 loci revealed any significant child-mediated genetic associations with ALL and no significant maternal

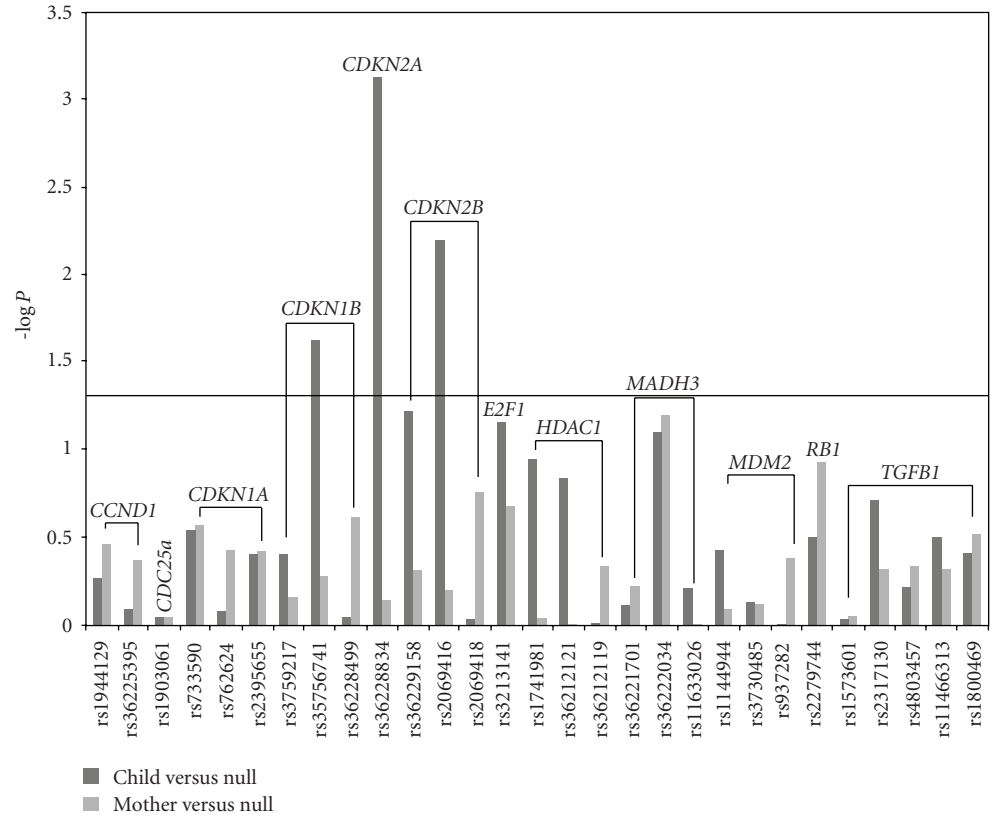

Figure 6: Log-linear, likelihood-ratio association analysis between 29 regulatory SNPs from 12 cell-cycle genes and childhood pre-B acute lymphoblastic leukemia (ALL). Log-linear regression analysis was performed in LEM using 203 case-triads, 118 unrelated ALL patients and 329 controls. Results of the likelihood-ratio chi-square tests ($-\log P$) are shown for the single-step Child (blue) and Mother (red) tests. Mating symmetry (i.e., six mating-type parameters) was assumed at all loci but variants rs1144944 and rs3730485 of the *MDM2* gene, as well as *CDKN2B* rs2069416 for which MA models (nine mating-type parameters) were used to test for association. Horizontal reference line indicates P value of .05. Note that for the tri-allelic variant *CDKN2B* rs2069416 (A > T,C) individuals were grouped according to their T allele such that $^{**} > {}^{*}\mathrm{T} > \mathrm{TT}$. See Supplementary Table 1 for complete likelihood-ratio chi-square test results.

genotype effects were identified through the Mother versus Null log-linear test (Figure 6 and Supplementary Table 1).

Nominally significant SNPs were further analyzed to detect putative joint fetomaternal effects. After accounting for the effect of the child's genotype, we found a significant maternal association at *CDKN2A* rs36228834 (Child + Mother versus Child; $P = .0168$) only (Supplementary Table 1). The *CDKN2A* rs36228834 A allele was overrepresented in patients when compared with controls (genotype Fisher's exact $P = .005$) and carriers of the A risk allele were 2.5-fold more susceptible to ALL (Child versus Null; TA versus TT: OR = 2.48; 95%CI (1.45–4.15); TA/AA versus TT: OR = 2.56; 95%CI (1.54–4.26)). This risk was further increased when the mother's genotype was included in the model (Child + Mother versus Null; TA versus TT: OR = 3.13; 95%CI (1.81–5.40); TA/AA versus TT: OR = 3.20; 95%CI (1.85–5.53)) (Table 5). No further maternal association was detected for *CDKN1B* rs35756741 (Child + Mother versus Child; $P = .59$) or *CDKN2B* rs2069416 (Child + Mother versus Child; $P = .33$) (Supplementary Table 1). These results provide evidence of a novel fetomaternal effect at the *CDKN2A* rs36228834 locus that may influence pre-B ALL susceptibility among children, and a possible joint effect of both mother and child genotypes without main effects at *CDKN2B* rs36229158. Interestingly, although variant *CDKN2B* rs36229158 presented only a suggestive effect at the level of the child (Child versus Null; $P = .06$), a significant association was found for the mother after we accounted for the genotype of the child (Child + Mother versus Child; $P = .0217$) (Supplementary Table 1). Including the mother's genotype in the regression model significantly increased the risk 2.3-fold for carriers of a C allele (Child + Mother versus Null; CT versus CC: OR = 2.32; 95%CI (1.23–4.35); CT versus CC: OR = 2.44; 95%CI (1.29–4.60)) (Table 5).

Independent replication is required in order to confirm the significance of these associations given that some of these variants did not withstand multiple testing correction (*CDKN1B* rs35756741 and *CDKN2B* rs36229158); and caution is warranted in the interpretation of the risk estimates as risk allele frequencies at loci *CDKN2A* rs36228834 and

CDKN2B rs36229158 were low (MAFs ≤ 0.05), yielding large confidence intervals, particularly for the rare homozygous genotype classes. We also recognize that the interpretation of our real-data results relies in part on our simulation results and there are certain limitations to our simulation study. These include the restricted number of models used in the method evaluations and the important assumptions of absence of population substructure and Hardy-Weinberg equilibrium, which necessitate further investigation. Our conclusions on the validity of the HD-NPC approach under MA should hold for other models. As we noted, the detection of asymmetry and maternal effects are partially confounded with HD-NPC and it is difficult to imagine a model of asymmetry for which differences in mating type frequencies would not be detected by the asymmetry test but would be captured by the Mother versus Null test, thus leading to increased type I error. However, the reduction in power of HD-NPC compared to HD will likely be affected by the underlying genetic and asymmetry models and should be assessed under a wider range of models. Other important genetic effects should also be investigated, such as mother-gene child-gene interactions and parent-of-origin effects which are not addressed here but can also be involved in early-onset disorder risk.

Nonetheless, our results provide evidence that genes that regulate the cell cycle could play an important role during fetal development when the rate of cell growth and division is high both in child and mother. *In silico* analysis using the Match software [37] revealed that all three variant loci lead to the disruption of putative transcription factor binding sites, including the loss of binding sites for FOS and MYB at *CDKN2A* rs36228834. The FOS oncoprotein stimulates transcription of genes containing AP-1 regulatory elements and may transform cells through alterations in DNA methylation and in histone deacetylation [38]. Expression of FOS is 100-fold greater in human fetal membranes than in other normal human tissues and cells [39]. The MYB transcription factor is essential for hematopoiesis and controls the proliferation and differentiation of hematopoietic stem and progenitor cells [40]. MYB is frequently involved in hematopoietic disorders including ALL [41]. Although the biological relevance of our findings remains to be elucidated, our study suggests that promoter variation in the cell-cycle inhibitor gene *CDKN2A*, and possibly *CDKN2B*, could disrupt transcription-factor binding and influence gene expression during gestation. Disregulated cell division caused by aberrant cell-cycle inhibitor gene expression in both mother and child could disrupt the maternal-fetal interface and affect important physiological processes such as the growth of the fetus and/or normal haematopoiesis and potentially lead to increased susceptibility to ALL.

4. Conclusions

Given the unique nature of childhood disorders, the investigation of parental genetics and maternally-contributed effects is a prerequisite not only for understanding disease etiology but also to pave the way toward new opportunities in preventive medicine. Although the most powerful approach is ideally the most desirable, in practice the best approach might be one that combines both valid detection of the possible underlying genetic associations involved in early-onset disorders and a feasible design in terms of ascertainment and genotyping costs. We have shown that the log-linear, likelihood-based framework using a case-triad/case-control design retains the ability to control for bias due to MA and can provide valid tests for maternally-contributed genotype effects even when the assumption of symmetry fails. Despite a modest sample size, we successfully used this approach to identify putative fetomaternal genotype effects in cell-cycle inhibitor genes *CDKN2A* and *CDKN2B* that are associated with modified risks of childhood pre-B ALL. Although these genes have been previously associated with ALL [28], we have shown for the first time that their influence on ALL risk might be driven, in part, by the maternal genotype. This study provides the first indication that maternal genotype effects can influence the risk of developing pediatric ALL, providing useful insights into the genetic mechanisms underlying this early-onset disease.

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Research Article

Association of Combined Maternal-Fetal *TNF-α* Gene G308A Genotypes with Preterm Delivery: A Gene-Gene Interaction Study

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Preterm delivery (PTD) is a complicated perinatal adverse event. We were interested in association of G308A polymorphism in *tumor necrosis factor-α* (*TNF-α*) gene with PTD; so we conducted a genetic epidemiology study in Anqing City, Anhui Province, China. Case families and control families were all collected between July 1999 and June 2002. To control potential population stratification as we could, all eligible subjects were ethnic Han Chinese. 250 case families and 247 control families were included in data analysis. A hybrid design which combines case-parent triads and control parents was employed, to test maternal-fetal genotype (MFG) incompatibility. The method is based on a log-linear modeling approach. In summary, we found that when the mother's or child's genotype was G/A, there was a reduced risk of PTD; however when the mother's or child's genotype was genotype A/A, there was a relatively higher risk of PTD. Combined maternal-fetal genotype GA/GA showed the most reduced risk of PTD. Comparison of the LRTs showed that the model with maternal-fetal genotype effects fits significantly better than the model with only maternal and fetal genotype main effects (log-likelihood = −719.4, $P = .023$, significant at 0.05 level). That means that the combined maternal-fetal genotype incompatibility was significantly associated with PTD. The model with maternal-fetal genotype effects can be considered a gene-gene interaction model. We claim that both maternal effects and fetal effects should be considered together while investigating genetic factors of certain perinatal diseases.

1. Introduction

Preterm delivery (PTD) is one of the main causes of perinatal and neonatal death. It has been reported that PTD is associated with some severe complications, such as cerebral palsy, chronic respiratory illness, and blindness [1]. Even in some developed countries like the United States, more than 10% of newborns are preterm, and the PTD prevalence is still increasing [2–4]. Genetic factors may be important determinants of PTD because women who were born preterm are more likely to deliver preterm; approximately 20% of women who delivered preterm subsequently had another PTD with the same partner; to change partners reduces the risk of PTD by one third and twin studies of pregnancy outcomes estimated the heritability of PTD as 17% to 36% [5].

Increasing clinical and laboratorial evidence suggests that amniochorionic-decidual infections may play an important role in PTD, by triggering a cascade of events that result in both spontaneous preterm labor (PTL) and preterm premature rupture of membranes (PPROMs). Researchers become interested in proinflammatory cytokines like tumor necrosis factor-α (*TNF-α*). *TNF-α* is a potent cytokine which has a wide range of proinflammatory activities [6]. Production of *TNF-α* gene is regulated partly at transcriptional level. An SNP from a normal guanine (G) allele to a variant adenine (A) allele at position 308 (G308A), which is located in the promotor region of *TNF-α*, is of particular interest.

Transfection studies indicated that *TNF-α* expression is higher in the presence of the −308A allele, compared with the −308G allele [7]. The G308A transition of *TNF-α* has been shown increasing both *TNF-α* concentration [8] and disease susceptibility in human subjects [9].

Large-scale studies of the association of the G308A polymorphism of *TNF-α* with PTD have been conducted only recently. However, no strong convincing evidence of association has been found. A systematic review has been reported, which reviewed studies investigating the association of the G308A polymorphism of *TNF-α* with PTD [10]. Those studies were reported between 1990 and 2005. Among the total seven studies involved in meta-analysis, only two reported positive results. A meta-analysis of the pooled dataset showed no statistically significant association. Trying to make progress, some researchers have tested population stratification as a potential confounder [11–13], and some have considered high-dimensional gene-gene interactions [14].

It is well known that pregnancy is a complicated course, depending on the balance between the mother and the fetus. Maternal-fetal incompatibility is thought to be one potential mechanism of adverse pregnancy outcomes. Therefore, while investigating certain perinatal diseases, it is recommended that one takes both maternal effects and fetal effects into consideration. Somewhat disappointingly, few studies have addressed association of G308A polymorphism of *TNF-α* with PTD in this way. In the current study, we used a hybrid design which combines case-parent triads and control parents in the data analysis [15], to explore the complicated effects of *TNF-α* G308A polymorphism on PTD. This hybrid design can bring the strengths of family-based designs and population-based designs together to test for maternal-fetal genotype (MFG) incompatibility, which can be considered a form of interaction between maternal genotypes and fetal genotypes.

2. Materials and Methods

2.1. Study Site and Population. Our study was conducted in Anqing City, Anhui Province, China. The city stretches about 80 km along the north bank of Yangtze River and includes eight counties. The total population of Anqing in 2000 was 6.8 million, with 20% of it living in urban areas. The birth rate was 15.1 per 1,000 people and the infant mortality rate was 3.8 per 1,000 live births.

Case families and control families were collected in Anqing Hospital between July 1999 and June 2002. Infants and their parents were all enrolled. Cases were defined as singleton, live, preterm infants (28 completed weeks or more but less than 37 completed weeks of gestation, regardless of birth weight); controls were defined as singleton, live, term infants (more than 37 completed weeks of gestation). Infants with birth defect were excluded. We matched cases and controls by maternal age (within 5 years) and delivery date (within 2 days). To control potential population stratification as we could, all enrolled subjects were ethnic Han Chinese. Besides, we enrolled only spontaneous PTD, to reduce heterogeneity within the case group to some extent.

2.2. Data Collection Procedures. All eligible mothers were approached by trained examiners soon after the delivery of their children. After informed consent (approved by the Ethics Committee of the Peking University Health Science Center) was obtained, a structured interview was conducted to obtain relevant information on demographic characteristics, cigarette smoking, alcohol consumption, as well as medical and reproductive history. Medical records of mothers and infants were reviewed to obtain clinical data including prenatal care, pregnancy complications, and birth outcomes (infant's gender, gestational age, and birth weight).

2.3. Blood Sample Collection and DNA Extraction. Cord blood samples (10 mL) from infants and peripheral blood samples (10 mL) from parents were collected. The blood samples were initially stored in a −20°C designated refrigerator in the *Labor and Delivery Ward* of the hospital and then transported to Peking University Health Science Center, via cold chain. DNA was extracted in our laboratory according to standard protocols [16].

2.4. Genotyping Methods. *TNF-α* is located in Chromosome 6p21.3, within the major histocompatibility gene complex [17]. We analyzed G308A polymorphism according to a reported method [18]. Primers used for PCR were 5′-GGGACACACAAGCATCAAGG-3′ and 5′-AATAGGTTT-TGAGGGCCATG-3′. After an initial denaturation step for 1 minute at 94°C, 40 cycles of amplification were performed as follows: 15 s at 94°C, 30 s at 60°C, and 15 s at 72°C followed by a final extension step for 7 minutes at 72°C. We used the Gene-Amp PCR System 9700 (Perkin-Elmer, Foster City, CA) for amplification. PCR products were digested with NcoI at 37°C. After ethidium bromide (EB) staining, diagnostic fragments were electrophoresed in 4% agarose gel and then visualized by UV transillumination. Genotyping accuracy was checked by duplicate genotyping within 10% random samples. Direct sequencing of PCR products was also performed to ensure genotyping results.

2.5. Statistical Methods. Our analysis employed the hybrid design reported by Weinberg and Umbach [15] as well as the ideas of testing maternal-fetal genotype (MFG) incompatibility reported by Sinsheimer and her colleagues [19]. For detailed information, please refer to the original papers.

The MFG test is based on a log-linear approach using only case-parent triads [20], while the hybrid design using both case-parents triads and control parents. Three key assumptions should be considered: Mendelian transmission of alleles, no population stratification, and mating symmetry. Mendelian transmission of the variant allele can be tested by *Transmission Disequilibrium Test (TDT)*, using control-parent triads. Because the MFG test employs only case-parent triads, mating symmetry cannot be tested [15, 21]. If one cannot trust the mating symmetry assumption, the appropriateness of using the MFG test is in doubt. The hybrid design provides ways of testing both the no population stratification and mating symmetry assumptions.

Luckily, even when one encounters mating asymmetry, a modification of the hybrid design using 9 ordered mating-types provides an alternative inference framework.

We first counted case-parent triads and control-parent triads based on six mating-types. After testing Mendelian transmission, we used the methods of testing no population stratification assumption and mating symmetry assumption proposed by the hybrid design.

Equation (1) is the standard alternative model presented by Weinberg and Umbach:

$$\begin{aligned}&\ln[E(\text{count} \mid M, F, C, D)]\\ &= \ln\left(\mu_j\right) + \beta_1 DI_{(C=1)} + \beta_2 DI_{(C=2)}\\ &\quad + \alpha_1 DI_{(M=1)} + \alpha_2 DI_{(M=2)} + \gamma D + \ln(\text{Off}).\end{aligned} \tag{1}$$

When $\beta_1 = \beta_2 = 0$, there are no child allelic effects in the model; when $\alpha_1 = \alpha_2 = 0$, there are no maternal allelic effects. Both of these restrictions hold under the null model. M, F, and C donate the number of copies (0, 1, or 2) of the variant allele carried by the mother, the father, and the affected child, respectively. D is an indicator variable that equals 1 for families with an affected offspring and 0 for control families. μ_j ($j = 1,\ldots,6$) are proportional to the relative frequencies in the population for the mating-type categories. β_1, β_2, α_1, and α_2 are relative risk parameters. "Off" is the probability multiplier (1, 1/2, or 1/4) for the particular cell (see Table 1 of [15]). $I_{(C=1)}$ denotes a dummy independent variable that equals 1 when $C = 1$ and 0 otherwise. It is similar for $I_{(C=2)}$, $I_{(M=1)}$, and $I_{(M=2)}$.

Augmenting (1) with an predictor $(M + F)D$ and testing for improvement in fit enables one to test whether there is bias due to population structure. Adding an interaction term between $I_{(M>F)}$ and mating-type ($I_{(M>F)}$ is defined as an indicator variable which is 1 when $M > F$ and 0 otherwise), one can test mating symmetry. Retaining any subset of (1) enables one to compare models with or without selected terms. We followed the ideas of the MFG test to test maternal-fetal genotype incompatibility by reparameterizing (1) with 6 maternal-fetal genotype relative risk combinations where the situation that both the mother and child have genotype G/G is considered the reference state. The *Likelihood Ratio Test (LRT)* is employed in model comparison. To ease understanding the five models we used in the current study, we present the models below symbolically.

Model I serves as a reference model and only includes 6 mother-father mating-type effects and disease effects:

$$\ln[E(\text{count} \mid M, F, C, D)] = \text{In}\left(\mu_j\right) + \gamma D + \ln(\text{Off}). \tag{I}$$

Model II has only fetal genotype effects, assuming no maternal genotype effects:

$$\begin{aligned}&\ln[E(\text{count} \mid M, F, C, D)]\\ &= \ln\left(\mu_j\right) + \beta_1 DI_{(C=1)} + \beta_2 DI_{(C=2)} + \gamma D + \ln(\text{Off}).\end{aligned} \tag{II}$$

Model III has only maternal genotype effects, assuming no fetal genotype effects:

$$\begin{aligned}&\ln[E(\text{count} \mid M, F, C, D)]\\ &= \ln\left(\mu_j\right) + \alpha_1 DI_{(M=1)} + \alpha_2 D\text{I}_{(M=2)} + \gamma\text{D} + \ln(\text{Off}).\end{aligned} \tag{III}$$

Model IV is (1).

Model V includes the combined maternal-fetal genotype effects instead of maternal genotype main effects and fetal genotype main effects:

$$\begin{aligned}&\ln[(\text{count} \mid M, F, C, D)] =\\ &\ln\left(\mu_j\right) + \delta_1 DI_{(M=2,C=2)} + \delta_2 DI_{(M=2,C=1)} + \delta_3 DI_{(M=1,C=2)}\\ &\quad + \delta_4 DI_{(M=1,C=1)} + \delta_5 DI_{(M=1,C=0)} + \delta_6 DI_{(M=0,C=1)}\\ &\quad + \gamma D + \ln(\text{Off}).\end{aligned} \tag{V}$$

The hybrid design employs case-parent triads and control parents, but not controls. So the genotypes of controls in the later analysis are treated as missing. Under this circumstance, the expectation-maximization (EM) algorithm is used to estimate relative risk parameters. The calculation can be conducted in the LEM software (log-linear and event history analysis with missing data using the EM algorithm), which was developed by Vermunt [22]. To aid us in the somewhat complicated calculation of EM, Weinberg and Umbach have provided LEM scripts in their website [15].

3. Results

A total of 520 families, including 260 control families and 260 case families, participated in our study. The participation rate was 90% among the control group and 95% among the case group. We tested maternal ethnicity or other sociodemographic characteristics between participants and nonparticipants and found no significant differences. Four case families and five control families were excluded because we lacked their blood samples; six case families and eight control families were excluded because of genotyping failures. Reproducibility of genotyping was routinely greater than 99%. We didnot encounter any cases of Mendelian inconsistency. The final analysis included 250 case families and 247 control families.

Table 1 shows some general characteristics of mothers and children.

Table 2 shows the 6 mating-types and their corresponding counts, for control group and case group, respectively. In the test of Mendelian transmission of the variant allele, we found no statistical evidence of non-Mendelian transmission ($\chi^2 = 0.42$, $P = .522$).

Although we have genotypes of controls as well, to suit the hybrid design, we rearranged data in Table 2 to form 24 possible cells, just as described in [15, Table 1]. For simplicity of the context, we didnot provide another table here.

In the LRT testing whether there is bias due to population stratification, we could not reject the null hypothesis of no

Table 1: Some general characteristics of mothers and children.

Variable	Control Group	Case Group	OR	Preterm Delivery 95%CI		*P*-value
Age, year						
19-	74	72	1	—	—	—
23-	99	105	1.09	0.72–1.67		.679
27-	74	73	1.01	0.64–1.60		.953
Education						
≤ elementary school	75	70	1	—	—	—
= middle school	133	145	1.18	0.79–1.75		.431
≥ middle school	39	35	0.95	0.54–1.66		.857
Occupation						
Farmer	110	111	1	—	—	—
worker	51	51	0.98	0.62–1.57		.935
Housewife	86	88	1.01	0.68–1.50		.955
Parity						
No	171	158	1	—	—	—
Yes	76	92	1.28	0.89–1.86		.187
Children gender						
Male	108	121	1	—	—	—
Female	139	129	0.82	0.58–1.17		.282

Table 2: Triad counts in control group and case group by genotype.

Mating-type[#]	Triad genotype (MFC)*	Control Group (N = 247)	Case Group (N = 250)
1	222	2	0
2	212	2	3
2	211	2	6
2	122	0	1
2	121	4	0
3	201	4	1
3	021	0	0
4	112	4	4
4	111	4	1
4	110	4	5
5	101	13	3
5	100	6	0
5	011	6	1
5	010	4	1
6	000	192	224

[#] Mating-type: decided by the number of copies of allele the parents carrying.
*Triad genotype (MFC): copies of the variant in mother, father, and child. 0: homozygous wild type; 1: heterozygous variant type; 2: homozygous variant type.

population stratification at the 0.05 significance level (χ^2 = 3.13, P = .077). However in testing mating symmetry, we detected significant evidence of violation (χ^2 = 11.93, P = .008). Therefore, we conducted our analyses with nine ordered parental genotype categories instead of the usual six. That is, for mating-types 2, 3, and 5, we divided them into two ordered mating-types each.

Table 3: LRTs for maternal and fetal genotype effects and their interaction.

Model[#]	*TNF-α G308A*	
	Log-likelihood*	*P* value**
I	−728.3	—
II	−724.8	.030[†]
III	−723.0	.005[†]
IV	−722.0	.013[†]
V	−719.4	.023[§]

[#]: Model I: with mating-type only; model II: with mating-type plus fetal genotype; model III: with mating-type plus maternal genotype; model IV: with mating-type plus fetal genotype and maternal genotype; model V: with mating-type plus combined maternal-fetal genotype.
*: The log-likelihoods were estimated using the LEM software.
**: LRTs, for a χ^2 distribution with df equal to the difference in the number of parameters being fitted.
[†]: Compared with Model I.
[§]: Compared with Model IV.

Table 3 shows the LRT results for the various models where model I serves as a reference. Comparing model II to model I, we can test for fetal genotype effects, assuming no maternal genotype effects. Comparing model III to model I, we can test for maternal genotype effects, assuming no fetal genotype effects. Comparing model IV to model I tests for both maternal genotype effects and fetal genotype effects simultaneously. Finally comparing model V to model IV tests for maternal-fetal genotype incompatibility and is a test of gene-gene interaction.

Table 4 shows the estimated relative risks of PTD, given maternal and fetal *TNF-α* G308A polymorphism. In summary, all the estimated relative risks of maternal genotype main effects and fetal genotype main effects were

Table 4: Associations of maternal and fetal *TNF-α* gene G308A genotypes with preterm delivery.

TNF-α G308A	Child		Mother	
Genotype*	RR	95%CI	RR	95%CI
GG†	1.0	—	1.0	—
GA	0.58	0.23–1.42	0.46	0.20–1.04
AA	0.93	0.28–3.04	1.23	0.34–4.46

*: GG: homozygous wild type; GA: heterozygous variant type; AA: homozygous variant type.
†: Reference category.

Table 5: Association of combined maternal-fetal *TNF-α* G308A genotypes with preterm delivery.

Genotypes*		Preterm delivery	
Child	Mother	RR	95%CI
GG	GG†	1.00	
GG	GA	0.36	0.14–0.96§
GA	GG	0.25	0.03–1.95
GA	GA	0.20	0.07–0.58§
GA	AA	1.12	0.41–3.10
AA	GA	0.82	0.30–2.24
AA	AA	0.59	0.15–2.25

*: Use LRTs, comparing the model with an interaction term for combined maternal-fetal genotypes against the model with maternal genotype and fetal genotype only (without interaction term).
†: Reference category.
§: Significant.

not significant (95%CI of RR includes 1). Nevertheless, mothers carrying 1 copy of the variant allele A had reduced risk, which was almost significant.

Table 5 presents estimated relative risks of PTD, given certain combined maternal-fetal genotypes. Combined maternal-fetal genotypes of GA/GG (RR = 0.36, 95%CI = 0.14–0.96) and GA/GA (RR = 0.20, 95%CI = 0.07–0.58) showed significant reduced risk of PTD.

4. Discussion

Because most reported studies investigating association of *TNF-α* G308A polymorphism with PTD didnot consider maternal effect and fetal effect together, we conducted the current study. We were interested in the combined maternal-fetal genotype effects on PTD. We used a hybrid design that combines case-parent triads and controls parents in the data analysis. Assumptions of Mendelian transmission of the variant allele and no population stratification were satisfied, but mating asymmetry was detected. So we conducted the following analysis based on models with nine ordered parental genotype categories instead of the usual six. Despite some loss of power, this hybrid design provides a still-valid analytic framework for inference, especially for maternal genetic effects [15].

Some of our results were insignificant, which may result from sparse data. Nevertheless, compared to heterozygote of variant allele A, homozygote of variant allele A showed relatively higher risk of PTD (see Table 4). This is in accordance with some aspects of our current understanding of the underlying biological processes. The balance between pro- and anti-inflammatory cytokines is critical for implantation, placental development and pregnancy outcome. While the T helper 1 (Th1) cytokine is associated with inflammation, the T helper 2 (Th2) cytokine is associated with anti-inflammation. The predominant expression of Th2 cytokine is likely to be important to reduce aberrant inflammation and allograft rejection of the fetus [23].

Chorioamnionitis presents to most PTD [24]. It is known that chorioamnionitis with high-grade leukocyte infiltration usually indicates intrauterine infection [25, 26], accompanied by high concentrations of inflammatory mediators in amniotic fluid like proinflammatory cytokines (such as *TNF-α*) [27, 28]. The G308A transition in *TNF-α* promotor region can increase gene expression [29, 30]. When inflammation does happen, an elevated proinflammatory cytokines level may result in changes of Th1/Th2 cytokine profile at the fetal-maternal interface, so as to break down the status of the pregnant uterus as an immune-privileged organ and cause different adverse consequences [31]. Even if no infection exists, proinflammatory cytokines can transform the uterus from a quiescent to an active state. The cytokines stimulate uterine activity via production of uterine activation proteins (UAPs) [32]. An active status of the uterus increases the possibility of PTD. These may account for why the G308A transition increases the risk of PTD.

The effect of G308A transition is far more complicated. There is one result that needs to be mentioned: mothers and children whose genotypes are heterozygous A/G lead to reduced risk of PTD (see Table 4). Similar phenomenon can be seen in the result of testing maternal-fetal genotype incompatibility (see Table 5). Combined maternal-fetal genotype GA/GA showed the most reduced risk of PTD, and this result was significant. One possible explanation is there are some cell membrane-bound molecules expressed in tissues of the fetus (like cytotrophoblasts), such as Fas ligand (FasL, also called CD95L) and *TNF*-related apoptosis-inducing ligand (TRAIL) [33]. Proinflammatory cytokines like *TNF-α* can upregulate their expression, enhance their function, or downregulate counteracting elements against them, which favor the apoptosis of infiltrated inflammatory cells [34, 35]. Moderate extent of these processes might benefit pregnancy, which the genotype G/A might induce. But of course, on the other side of the coin, if these processes are too strong, an active status of the uterus ensues and inevitably increases the possibility of PTD, which the genotype A/A might induce.

Maternal genotype effects have long been involved in the study of perinatal diseases. In recent years, there have been emerging hypotheses and evidence that the fetal genome might be associated with pregnancy health, either by themselves or by interacting with maternal genes. The failure to recognize fetal contributions to pregnancy health may be at least partially responsible for the inability to consistently identify predisposing maternal genetic variants [36]. Since 1999, more than ten studies have investigated association of the G308A polymorphism with PTD, but only three of them

have involved both mothers and children in their studies. Even for these three studies, none considered the mother and the child together in their modeling. In our study, we used a modified version of the hybrid design of Weinberg and Umbach in an LRT framework to test. The model with combined maternal-fetal genotype effects is favored (log-likelihood = −719.4, $P = .023$) over the model with just maternal and fetal genotype main effects. It implies that fetal genotype effects might contribute to PTD by interacting with maternal genotype effects. This kind of combined maternal-fetal genotype effects can be viewed as a special maternal-fetal gene-gene interaction [36]. Pregnancy is a complicated biologic course, and there is interplay between the mother and the fetus. The mother needs to employ immune-privileged processes to maintain a maternal-fetal balance, while the fetus needs to survive immune rejection. Regarding the result presented in Table 5, one may find that risk of PTD could not be predicted just simply by number of copies carried by the mother and/or the child. It is far more complicated. Combination of maternal-fetal genotypes, that is maternal-fetal genotype incompatibility, deserves more of our attention as a potential source of risk.

There are limitations in our study. First, although we have enrolled a moderate sample size of triads, relative small numbers of informative triads might influence the precise of our results. Zero counts of cells may undermine the power of LEM. This limitation partly resulted from low frequency of the variant allele in our study population. Variant allele frequency varies remarkable among different races. Since there are few large sample size studies which can provide reliable information of *TNF-α* gene −308A allele frequency in Han Chinese, further studies are warranted to confirm our results. Another limitation is that there was mating asymmetry in our data. This limitation might be somehow relevant to the first one. Under mating asymmetry, the standard MFG test [19] cannot be applied to our study. However the hybrid design could be modified to include MFG incompatibility and saved our study by providing a still-valid analytic framework for inference. Finally, PTD is likely to be etiologically heterogeneous. Although we enrolled only spontaneous PTD, we couldn't specify detailed types of PTD due to practical reasons. This aspect should be improved in future. Since we were interested in *TNF-α* which is a proinflammatory cytokine, and chorioamnionitis presents to most PTD, the influence of heterogeneity within the case group may be mild. No population stratification may also lessen this limitation.

5. Conclusion

In this study, we used a hybrid design and ideas of the MFG test studying maternal-fetal genotype incompatibility, to explore association of *TNF-α* G308A polymorphism with PTD. For the first time, as far as we know, we have reported an association of combined maternal-fetal *TNF-α* gene G308A genotypes with PTD. This association was consistent and significant. Besides, G308A transition resulted in relatively higher risk of PTD for those who are homozygous A/A, compared to those who are heterozygous A/G. Combined maternal-fetal genotype GA/GA showed the most reduced risk of PTD. But since there were zero counts in our data, the results should be interpreted with caution and our findings need be confirmed by similar studies.

Understanding the interaction between maternal and fetal genes is important when studying genetic factors of perinatal diseases. Of special interest, the statistical methods we used here are robust to the potential confounding that can occur when investigating this interaction and use both family-based data and population-based data. There are few studies like ours, and so our study is of importance despite the previously mentioned limitations.

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Review Article

Evidence for Maternal-Fetal Genotype Incompatibility as a Risk Factor for Schizophrenia

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Prenatal/obstetric complications are implicated in schizophrenia susceptibility. Some complications may arise from maternal-fetal genotype incompatibility, a term used to describe maternal-fetal genotype combinations that produce an adverse prenatal environment. A review of maternal-fetal genotype incompatibility studies suggests that schizophrenia susceptibility is increased by maternal-fetal genotype combinations at the *RHD* and *HLA-B* loci. Maternal-fetal genotype combinations at these loci are hypothesized to have an effect on the maternal immune system during pregnancy which can affect fetal neurodevelopment and increase schizophrenia susceptibility. This article reviews maternal-fetal genotype incompatibility studies and schizophrenia and discusses the hypothesized biological role of these "incompatibility genes". It concludes that research is needed to further elucidate the role of *RHD* and *HLA-B* maternal-fetal genotype incompatibility in schizophrenia and to identify other genes that produce an adverse prenatal environment through a maternal-fetal genotype incompatibility mechanism. Efforts to develop more sophisticated study designs and data analysis techniques for modeling maternal-fetal genotype incompatibility effects are warranted.

1. Introduction

Schizophrenia has long been regarded a significant public health issue. This condition, which is estimated to affect more than 2 million persons in the U.S. alone [1], has increased mortality and morbidity compared to the general population [2, 3]. Individuals with schizophrenia typically suffer from a combination of debilitating symptoms including hallucinations and delusions and treatment-resistant symptoms, such as social withdrawal [4]. The disease affects males and females, although there is evidence to support a number of sex differences in the characteristics of schizophrenia. Compared to females, males may be more likely to develop schizophrenia with ~1.4 : 1 ratio [5, 6], have an earlier age at onset [6–8], poorer premorbid social and intellectual functioning, poorer course and medication response, greater structural brain abnormalities [9], more negative, symptoms and fewer affective symptoms [10].

Twin, family, and adoption studies together suggest that schizophrenia is a complex disorder involving both genes and environment [11, 12]. Further, the evidence suggesting that schizophrenia arises from a process involving *prenatal* environmental conditions is compelling [13–21]. Numerous case-control studies have demonstrated that individuals with schizophrenia are more likely to have been exposed to prenatal/obstetric complications than their unaffected siblings, normal controls, or psychiatric controls [22], and a meta-analysis of twelve twin studies [11] demonstrated that a nontrivial proportion of liability to schizophrenia can be accounted for by a common or shared environmental effect (11%; 95% CI 3%–19%). Because the environments of twins are most similar in utero, the role of common environment effects on liability for schizophrenia would most likely occur very early in life. Suspected fetal environmental risks include exposure to maternal stress [23], influenza [24–26], infection [27], famine or prenatal nutritional deficiency [16, 28–31], and obstetric complications [14, 15, 18, 19, 32]. Several reviews [8, 21, 33], including a meta analysis [19], support the involvement of (i) pregnancy complications such as Rhesus D incompatibility and pre-eclampsia,

(ii) abnormal fetal growth and development, and (iii) delivery complications that produce fetal hypoxia as risk factors and suggest that obstetric complications contribute approximately a 2-fold increased risk for schizophrenia. Prenatal/obstetric complications are believed to disrupt normal fetal neurodevelopment and their involvement in schizophrenia susceptibility is consistent with the neurodevelopmental hypothesis of schizophrenia. This hypothesis posits that brain development is disrupted early in life and that subsequent maturational events in combination with other environmental factors leads to the emergence of psychosis during adulthood [34–36].

Support for the important role of prenatal/obstetric complications in schizophrenia also comes from neuroimaging studies. As an example, there is evidence that fetal hypoxia has a differential effect on the hippocampus of schizophrenics and their first degree relatives, suggesting that this temporal lobe region may be sensitive to prenatal environmental conditions [37–39]. Furthermore, anatomical deficits in the medial temporal lobe structures are more severe among patients with schizophrenia who have a history of hypoxia-associated obstetric complications [40]. Hence, not only do these studies suggest that factors that produce prenatal/obstetric hypoxia have an effect on the medial temporal lobe structure, but that genetic liability to schizophrenia also plays a role in predisposing an individual to schizophrenia. This evidence has produced a variety of hypotheses regarding how genetic and environmental influences aggregate to increase susceptibility to schizophrenia, with gene-environment interaction, gene-environment covariation, or direct environmental effects, that is, phenocopy model, as (potentially overlapping) models [41]. To date, there is very little evidence to support a phenocopy model or a gene-environment covariation model to explain the role of prenatal/obstetric complications in schizophrenia, although additional investigation of these models is warranted [41]. In contrast, evidence that prenatal/obstetric complications increase risk for schizophrenia through a gene-environment interaction model is accumulating. In addition to the studies cited earlier [37–40], a recent study found that risk of schizophrenia was greatest among individuals at highest familial liability who were exposed to maternal infection (consistent with an interaction model) [27]. As another example, significant interaction between suspected hypoxia-regulated/vascular-expression genes and serious obstetric complications (predominantly hypoxia) was found to influence risk for schizophrenia [42].

Although the causes for prenatal complications are quite heterogeneous, their diversity does not exclude a final common pathway and there is increasing discussion that the common pathway involves both the immune and vascular systems in the pathogenesis of schizophrenia [43, 44]. In an excellent review of this theory of schizophrenia, Hanson and Gottesman [43] describe a process in which ubiquitous environmental factors that normally trigger genetically-influenced inflammatory response (infection, trauma, hypoxia) in individuals will trigger abnormal inflammatory processes in individuals with particular genotypes at these inflammatory response loci which results in damage to the microvascular system in the brain. This vascular-inflammatory theory not only accommodates the diversity of prenatal complications associated with schizophrenia, but also specifies an interaction between genes and environment. The latter point helps to explain why most people who experience prenatal/obstetric complications do not eventually develop schizophrenia [19, 21], and has received empirical support through the increasing number of studies demonstrating gene-environment interactions in schizophrenia [27, 37–40, 42], particularly the recent study that identified an interaction between serious obstetric complications and hypoxia-regulated/vascular-expression genes [42].

Not surprisingly, there remains considerable interest in identifying fetal environmental risk factors and elucidating their role in schizophrenia. However, in addition to their heterogeneity, prenatal/obstetric complications can be difficult to document reliably through medical records or maternal recall, making it difficult to test the role of environmental insults in the pathogenesis of schizophrenia. Of interest, there is some evidence that prenatal complications that increase susceptibility to schizophrenia cluster within schizophrenia families [45], which raises the possibility that some of these complications may have a genetic basis and that these risk genes and hence, adverse prenatal environment, can be measured directly through genetic analyses rather than through medical records or maternal recall. A benefit of direct measurement of the adverse prenatal environment through genetic analysis is that it can facilitate hypothesis testing regarding the role of prenatal/obstetric complications in the pathogenesis of schizophrenia.

Maternal-fetal genotype incompatibility, first described by Palmer et al. [47] to describe a mechanism that confers risk for schizophrenia through maternal-fetal genotype combinations which produce a maternal immunological reaction that creates an adverse prenatal environment, is an example of a prenatal/obstetric complication with a genetic basis. As will be described, maternal-fetal genotype incompatibility can occur when maternal and fetal genotypes differ from one another, or when maternal and fetal genotypes are too similar to each other. "Incompatibility genes" for each of these scenarios have been implicated as risk factors for schizophrenia and are reviewed below. Importantly, maternal-fetal genotype incompatibility is explicitly genetic in nature and so has the potential to be measured directly through genetic analyses even years after the adverse prenatal event has occurred.

2. *RHD* Maternal-Fetal Genotype Incompatibility as a Risk Factor for Schizophrenia: When Maternal and Fetal Genotypes Differ

The teratogenic antibody hypothesis [46] posits that a pregnant female can develop antibodies in response to some antibody producing stimulus (e.g., contact with paternal antigens) that can interfere with normal fetal neurodevelopment. One general mechanism that is consistent with

the teratogenic antibody hypothesis involves maternal-fetal genotype combinations that adversely affect the developing fetus by inducing a maternal immunological attack. This mechanism is a form of maternal-fetal genotype incompatibility [47], where the development of maternal antibodies can be the result of a mother's genotype that is different from the fetus' genotype.

In some cases, a maternal immunological reaction can lead to hypoxic ischemia, a condition found to be associated with schizophrenia [17, 18], and hypothesized to trigger abnormal inflammatory processes in individuals with vulnerable genotypes at inflammatory response loci resulting in damage to the micro-vascular system in the brain and increasing risk for schizophrenia [43]. Conditions that can produce fetal or neonatal hypoxia include maternal-fetal genotype incompatibilities at genes that produce red blood cell antigens, such as the *RHD* locus.

The *RHD* gene produces a red blood cell antigen called the Rhesus D factor. An individual who is determined to be Rhesus D positive has red blood cells (RBCs) with this antigen, while someone classified as Rhesus D negative lacks this antigen [48]. Individuals who are Rhesus D positive are either homozygous or heterozygous for an allele that produces the antigen (referred to here as D/D or D/d). Individuals who are Rhesus D negative are homozygous for a null allele (d/d). In Caucasian populations, approximately 85% of individuals are Rhesus D positive [48].

RHD maternal-fetal genotype incompatibility during pregnancy occurs when a pregnant woman is Rhesus D negative (d/d) and her fetus is Rhesus D positive (D/d). Because the RBCs of a Rhesus D negative pregnant female do not possess the Rhesus D antigen, maternal anti-D (IgG) antibodies are created in response to detection of fetal RBCs in the maternal blood stream [48]. These antibodies destroy the fetal RBCs in the maternal blood stream, cross the placenta, and destroy fetal RBCs. Because RBCs carry oxygen throughout the fetus' body, including the brain, an attack on the fetal RBCs increases risk for fetal hypoxia which could affect developing tissue, including brain tissue. A byproduct of the destruction of RBCs is bilirubin; thus hyperbilirubinemia, or jaundice can occur, as well as kernicterus, which is deposition of bilirubin in the brain [49]. Bilirubin is a known neurotoxin [50, 51] to which undifferentiated glial cells are sensitive [52, 53], and glial cell abnormalities also have been associated with schizophrenia [54, 55].

An infant is said to have Rhesus hemolytic disease of the newborn when clinical complications arise due to the *RHD* maternal-fetal genotype incompatibility. Because maternal sensitization usually does not occur until delivery of the first *RHD* incompatible pregnancy, it is not until second- and later-incompatible pregnancies that risk for a maternal immune attack becomes appreciable [48]. Around 1970 prophylaxis against maternal isoimmunization became available [56], which has made a dramatic impact on the morbidity and mortality associated with *RHD* maternal-fetal genotype incompatibility. However, even in an era of prophylaxis, there continue to be cases of Rhesus hemolytic disease of the newborn, either due to lack of prophylaxis use [56, 57], or because its use is not 100% effective at preventing maternal sensitization [58, 59].

Evidence to support involvement of *RHD* maternal-fetal genotype incompatibility in schizophrenia comes from both nongenetic and genetic studies performed on samples in which individuals with schizophrenia predominantly were born prior to 1970 [15, 19, 47, 60–66] and reviewed in [67]. The nongenetic studies are based on serotype data (Rhesus D negative, Rhesus D positive) or evidence of hemolytic disease of the newborn in mother-child pairs [19, 32, 60, 61, 63, 64, 66], while the genetic studies are based on genotype data (D/D, D/d, d/d) and nuclear families [47, 65, 67]. Collectively, these studies have provided evidence that *RHD* maternal-fetal genotype incompatibility is a risk factor for schizophrenia with relative risk ranging from 1.4 [32] to 2.26 [47], a magnitude, that is, comparable to the relative risk of schizophrenia due to obstetric complications in general [19] or due to most genes for which an association with schizophrenia has been observed (see [68, 69] for reviews). It is remarkable that studies of *RHD* maternal-fetal genotype incompatibility and schizophrenia that differ in design and population cohort should arrive at similar relative risk estimates; this consistency suggests that the relative risk of schizophrenia due to *RHD* maternal-fetal genotype incompatibility, although small, is substantively meaningful and worthy of more investigation.

Another way to look at the magnitude of the *RHD* maternal-fetal genotype incompatibility effect is to compute the population attributable fraction, that is, the number of cases which would not occur if the risk factor is eliminated. Based on formulas found in [70], for *RHD* maternal-fetal genotype incompatibility, the population attributable fraction is ∼3%, as estimated from the fraction of cases that have an *RHD* maternal-fetal genotype incompatibility (7.8% [65]) and the relative risk due to the incompatibility (using the most conservative estimate of 1.5 in [65]). Based on a population prevalence for schizophrenia of 1% and assuming that the allele frequencies for the *RHD* locus are homogeneous in the U.S., this attributable fraction suggests that more than 100,000 schizophrenia cases in the U.S. would not have occurred but for the *RHD* maternal-fetal genotype incompatibility. This is not a trivial number, and for comparison, it has been estimated that more than 100,000 cases of schizophrenia in the U.S. would not have occurred but for the val allele of the *COMT* gene [71]. Thus, these two loci, that is, *COMT* and *RHD*, could potentially account for an effect of similar size at the population level. Of course, the allele frequencies at the *RHD* locus differ across populations, with the d allele being less common in some populations than others (e.g., $P(\mathrm{D}) = .76$ in a study conducted in Nairobi [72] compared to $P(\mathrm{D}) = .66$ in the Finnish population [73]). Thus, the frequency of Rhesus negative mothers having Rhesus positive children will vary across populations. This variability in allele frequencies will not affect the relative risk of disease due to *RHD* maternal-fetal genotype incompatibility, but will affect the fraction of schizophrenia cases that are attributed to the *RHD* locus across populations.

Since most individuals who are exposed to *RHD* maternal-fetal genotype incompatibility do not develop schizophrenia, it is highly unlikely that exposure to this adverse prenatal environment alone, that is, a phenocopy model, explains risk for schizophrenia. Furthermore, the lack of evidence for violation of Hardy-Weinberg equilibrium in the founder alleles from the family-based *RHD* genetic studies [47, 67] is inconsistent with a gene-"environment" covariation model because it suggests that mate selection in the schizophrenia families occurred independently of *RHD* genotype, at least among the founders. To date there have been no empirical studies to determine whether the association between *RHD* maternal-fetal genotype incompatibility and schizophrenia is explained through a gene-"environment" interaction model.

There also is emerging evidence in studies based on serotype data and those based on genotype data that risk of schizophrenia due to *RHD* maternal-fetal genotype incompatibility may depend on offspring sex [61, 66, 67], with a relative risk of 1.64 in male incompatible offspring and 1.07 in female incompatible offspring based on a recent meta-analysis [67]. Furthermore, a nonsignificant trend suggesting that male offspring are at higher risk than female offspring for schizophrenia due to maternal-fetal genotype incompatibility at another RBC antigen locus, *ABO*, has also been identified based on serotype data [66]. *ABO* maternal-fetal genotype incompatibility occurs when a pregnant woman has type O blood and her fetus has type A or B [48]. As with Rhesus D incompatibility, maternal IgG antibodies can be produced against the fetal antigens and result in hemolytic disease of the newborn [74], although in this case the risk is the same for all pregnancies [75, 76].

These sex-dependent findings allow for hypotheses that address why the schizophrenia effect of an RBC antigen—associated maternal immune response is so much greater for male offspring compared to female offspring. It is unlikely to be the case that *RHD* maternal-fetal genotype incompatibility is more likely to occur in pregnancies with male offspring, nor is there evidence that its related condition of hemolytic disease of the newborn is more likely to occur in pregnancies with male fetuses compared to female fetuses. However, there is evidence that the clinical manifestations of *RHD* maternal-fetal genotype incompatibility are more severe in pregnancies with male fetuses than with female fetuses [77]. Thus one hypothesis is that specific schizophrenia-effects of *RHD* maternal-fetal genotype incompatibility (hypoxia, hyperbilirubinemia) can affect female fetuses but that they are less likely to surpass the threshold of severity compared to male fetuses (threshold effect).

There also is evidence that the clinical effects of *RHD* maternal-fetal genotype incompatibility may occur earlier in gestation for male fetuses compared to female fetuses [77]. Coupled with research supporting sex-differences in brain maturational rates [78], with males exhibiting a slower pace of cerebral development compared to females [7], another hypothesis is that male and females are equally vulnerable to the specific effects of *RHD* maternal-fetal genotype incompatibility, but that these effects must occur at sex-dependent times during development. This hypothesis further suggests that female fetuses may be at increased risk for schizophrenia when subject to prenatal/obstetric complications that produce hypoxia or hyperbilirubinemia, but that these effects must occur earlier in the gestational period to increase their risk of schizophrenia (a timing effect). A third hypothesis is that male fetuses, but not female fetuses, experience schizophrenia effects due to hypoxia or hyperbilirubinemia (a specific effect). Although there have not yet been studies addressing whether risk of schizophrenia due to *RHD* maternal-fetal genotype incompatibility in male and female fetuses is a function of a threshold effect or a timing effect, there have been studies addressing potential sex differences in rates of hypoxia-related in males and females, with conflicting results [79, 80].

The involvement of the *RHD* gene in the form of maternal-fetal genotype incompatibility as a risk factor for schizophrenia susceptibility is further substantiated by analyses that showed no evidence to support the idea that this locus is simply linked/associated with a nearby schizophrenia susceptibility locus or that this gene acts through the maternal genotype alone [47]. Furthermore, there is empirical evidence consistent with the hypothesized biological mechanism that *previous RHD* maternal-fetal genotype incompatible pregnancies increase risk for maternal isoimmunization in subsequent pregnancies in the two schizophrenia—*RHD* maternal-fetal genotype incompatibility studies that tested this hypothesis [61, 65]. Using serotype information, Hollister et al. [61] divided their birth cohort sample into firstborn Rhesus D-incompatible and Rhesus D-compatible males, and second- or later-born Rhesus D-incompatible and Rhesus D-compatible males. Consistent with a birth order effect, they found the rate of schizophrenia among the second- or later-born Rhesus D-incompatible males was significantly higher than the second- or later-born Rhesus D-compatible males (2.6% versus 0.8%, $P = .05$); but that there was no significant difference in the rate of schizophrenia between the firstborn Rhesus D-incompatible and D-compatible males ($P = .64$).

In the second study, Kraft et al. [65] tested hypotheses about a birth order effect using nuclear families with at least one individual with schizophrenia and *RHD* genotype data. The model in which there is increased risk only to second- or later-born incompatible children fit the data well, with a significant point estimate of 1.7 relative risk to second- or later-born incompatible children ($P = .014$). The other model that fit the data well assumed an increased risk for all incompatible children regardless of birth order; the point estimate for the relative risk of schizophrenia in this model was 1.5 (lower than the former model). However, one issue with the latter model is that it forced the risk to first-born incompatible children to be identical to risk to later-born incompatible children, and essentially produced an average relative risk across birth order groups. Since the relative risk was estimated at 1.7 in a model that assumed no risk to first-born, and then the relative risk was lowered to 1.5 in a model that averaged over all incompatible children, the authors concluded that the effect of including the first-born incompatible children was to artificially *lower* the relative risk relative risk estimates of

RHD maternal-fetal genotype incompatibility for the later-born children. It is important to note that neither study had information on pregnancies that did not go to full term, for example, spontaneous abortions. The potential effect of this lack of information is to misclassify some *RHD* maternal-fetal genotype incompatible individuals as first-born (and at very low risk from maternal sensitization) when in fact they were later-born and at heightened risk due to previous maternal sensitization. Such misclassification would serve to underestimate the difference between groups and bias results toward the null hypothesis of no birth order effect. In light of the challenges of truly examining a birth order effect with *RHD* maternal-fetal genotype incompatibility, it is striking that the two schizophrenia studies that chose to test birth order hypotheses found evidence in support of such an effect. However, further examination of this hypothesis is warranted due to the findings that suggest that the risk of schizophrenia associated with *RHD* maternal-fetal genotype incompatibility is limited to male offspring. In this case, one would expect to observe an increased risk among second- or later-born incompatible males, but not females. This hypothesis has not yet been tested.

The involvement of Rhesus D incompatibility in schizophrenia was initially provided by studies that inferred genotype status through serotype data. Importantly, the evidence from these non-genetic studies provided the impetus for conceptualizing maternal-fetal genotype incompatibility as a more general non-Mendelian mechanism involved in the etiology of complex disorders such as schizophrenia. The first candidate gene study to test the hypothesis of *RHD* maternal-fetal genotype incompatibility as a risk factor for schizophrenia [47] provided the proof of principle that this non-Mendelian mechanism can be tested with genotype data. Further, it facilitated the development of statistical methods and study designs based on a candidate gene approach and nuclear families for addressing hypotheses about the role of maternal-fetal genotype incompatibility in disease [65, 81–85]. Such innovations are important because, as illustrated in the next section, not all "incompatibility genes" can be inferred through serotype data.

3. *HLA-B* Maternal-Fetal Genotype Incompatibility as a Risk Factor for Schizophrenia: When Maternal and Fetal Genotypes Do Not Differ

Human leukocyte antigens (HLAs) play an important role in the control of immune responses [86] and there has long been a belief that HLAs play a role in schizophrenia susceptibility, although with conflicting results from genetic studies examining the hypothesis of a high risk allele acting through the affected individual's genotype [87]. Another way to conceptualize the role of HLA in schizophrenia susceptibility is to consider its role(s) in pregnancy. There is strong evidence for maternal recognition of paternally-derived fetal HLAs during pregnancy because maternal antibodies against these fetal antigens have been detected [88]. However, maternal recognition of paternally-derived fetal HLAs that differ from maternal HLAs is believed to be beneficial to implantation and maintenance of pregnancy because maternal antibodies to fetal antigens have been observed in a large number of healthy pregnancies. In contrast, *lack* of maternal recognition, which is the result of paternally derived HLAs that are not perceived as different from the maternal HLAs, may lead to adverse reproductive outcomes [88].

The underlying biological mechanism for poor reproductive outcomes is not yet known, however, an immunological intolerance hypothesis posits that *HLA* similarity between mother and fetus fails to stimulate an adequate maternal immune response that is necessary for proper implantation and maintenance of pregnancy [89]. There is some empirical evidence that situations where maternal sensitization would not occur, that is, HLA matching between couples or between mother and fetus, increases the risk of fetal loss [89–92], preeclampsia [93–96], low birth weight [97–100], newborn encephalopathy, and seizures [101]. Importantly, low birth weight and preeclampsia are complications that have been associated with schizophrenia [15, 19, 102–104]. The mechanism(s) by which low birth weight or preeclampsia increase risk for schizophrenia is not yet known. However, a current theory regarding preeclampsia hypothesizes that this condition gives rise to abnormal fetal blood flow that results in chronic fetal hypoxia or malnutrition [105] and both of these conditions are associated with schizophrenia [15, 16, 28–31, 102, 103]. Furthermore, preeclampsia involves a generalized inflammatory response in the mother as a result of the oxidatively stressed or hypoxic placenta [106], and inflammatory processes are hypothesized to damage the microvasular system of the brain [43, 44] and increase risk of schizophrenia [43].

Two additional lines of evidence implicate maternal-fetal HLA matching in schizophrenia. First, evidence supporting the relevance of HLA matching to neurodevelopmental disorders comes from a study that found that parents of children with autism were significantly more likely to share at least one *HLA-A*, *-B*, or *-C* antigen in common compared with parents of unaffected children [107]. Second, circumstantial evidence supporting the relevance of HLA matching specifically to schizophrenia comes from the literature on mate selection and the literature on olfaction in schizophrenia. Specifically, in the mate selection literature there is some evidence to support disassortative mate selection with respect to HLA loci [108–111] and that olfaction plays a role in this process [109, 110, 112]. However, studies of individuals with schizophrenia and their unaffected first degree relatives reveal impairments in olfaction [113–116]. Hence, mate selection in this subgroup of individuals may be less likely to be guided by the ability to "sniff out" a mate with HLA dissimilarity, and thus more likely to result in the construction of couples with HLA similarity for whom maternal-fetal HLA matching is more likely to occur.

Because risk of schizophrenia is associated with prenatal/obstetric complications, including preeclampsia and low birth weight, maternal-fetal HLA matching has been associated with these and other pregnancy/obstetric complications, maternal-fetal HLA matching has been observed

in another neurodevelopmental disorder, and maternal-fetal HLA matching may occur more frequently in families of individuals with schizophrenia for biological reasons, a candidate gene study was conducted to assess maternal-fetal genotype incompatibility, that is, matching, at the *HLA-A*, *-B*, and *-DRB1* loci as a risk factor of schizophrenia [117]. For this study, Palmer and colleagues hypothesized that maternal-fetal genotype incompatibility increased risk of schizophrenia through a general allele-matching phenomenon rather than through specific allele combinations. For each locus, mother and offspring were considered to match if the offspring's alleles were identical to the maternal alleles or if the offspring's alleles were a subset of the maternal alleles. In either of these cases, maternal sensitization to fetal antigens would not occur because they would be perceived to be the same as the maternal antigens. The maternal-fetal genotype incompatibility test for multiple siblings [65] was modified to accommodate analyses involving a general allele-matching phenomenon and missing parental genotypes [82]. There was no evidence for violation of Hardy-Weinberg equilibrium in the founder alleles, consistent with random mating with respect to these three loci. There was no evidence for *HLA-A* or *-DRB1* maternal-fetal genotype matching effect on schizophrenia. In contrast, there was significant evidence for an *HLA-B* maternal-fetal genotype incompatibility effect ($P = .01$) where inspection of the parameter estimates revealed that maternal-fetal genotype matching produced a higher risk for female offspring (1.74, 95% CI: 1.22–2.49) than for male offspring (1.11, 95% CI: 0.76–1.61). Of note, in the mate selection literature, *HLA-B* appears to be particularly influential [118].

As this is the first study to demonstrate an association between *HLA-B* matching and schizophrenia, much more research is needed to determine the mechanism through which this form of maternal-fetal genotype incompatibility increases risk for schizophrenia. One possibility is that *HLA-B* matching increases risk for adverse reproductive outcomes such as preeclampsia or low birth weight. This hypothesis could be tested by examining prenatal and birth records in a sample of females with schizophrenia stratified by *HLA-B* matching status, and comparing the rates of preeclampsia, low birth weight, and other pregnancy/obstetric complications between the two groups. It also currently is unclear why female offspring would be more vulnerable to effects of *HLA-B* matching than male offspring. One possibility is that female fetuses are more likely to survive the putative effects of *HLA-B* matching, such as preeclampsia [119] and hence to be observed in a study, than male offspring. Although the sex-dependent finding is intriguing in light of the work demonstrating that *RHD* maternal-fetal genotype incompatibility as a schizophrenia risk factor is limited to males [67], replication and investigation of hypothesized clinical manifestations of *HLA-B* matching (low birth weight, preeclampsia, other complications) are warranted because other published studies reveal conflicting results regarding sex differences in the rates of low birth weight and preeclampsia among individuals with schizophrenia [102, 104]. Because the firstborn child of a couple is at highest risk for preeclampsia, one could also seek further evidence in support of an *HLA-B* matching—preeclampsia relationship by testing for a birth order effect.

Future research must provide additional evidence for an association between *HLA-B* matching and schizophrenia, determine if there are clinical outcomes of *HLA-B* matching, for example, prenatal/obstetric complications, whether *HLA-B* matching increases risk through a phenocopy model or a gene-"environment" interaction model, or is simply associated through a gene-"environment" covariation model, and determine the basis for a sex-dependent risk. It will be particularly important to distinguish between a gene-"environment" covariation model and a gene-"environment" interaction model given the a priori basis for expecting higher rates of *HLA-B* matching in schizophrenia as a function of olfaction deficits.

4. Future Research

Based on this review, there are a variety of hypotheses that could be tested in future research to further elucidate the role of *RHD* and *HLA-B* maternal-fetal genotype incompatibility in schizophrenia. One important area of research would focus on conducting studies that add to the evidence that these maternal-fetal genotype incompatibilities are risk factors for schizophrenia. As examples, since *RHD* maternal-fetal genotype incompatibility is genetic in origin, one would expect more clustering of schizophrenia in families with *RHD* maternal-fetal genotype incompatibility than in schizophrenia families without *RHD* maternal-fetal genotype incompatibility. The same hypothesis holds for *HLA-B* maternal-fetal genotype incompatibility. If *RHD* maternal-fetal genotype incompatibility is a risk factor specifically for males, one would expect to observe that schizophrenia risk is associated with a birth order effect with male offspring exposed to *RHD* maternal-fetal genotype incompatibility, but not for female offspring. If *HLA-B* matching is involved in predisposition to pre-eclampsia, low birth weight, or other prenatal/obstetric complication, one would expect higher rates of these prenatal/obstetric complications in individuals with schizophrenia with *HLA-B* maternal-fetal genotype matching compared to those without *HLA-B* maternal-fetal genotype matching. If *HLA-B* matching is involved specifically in predisposition to pre-eclampsia, one would expect to observe that schizophrenia risk is associated with a birth order effect with female offspring exposed to *HLA-B* maternal-fetal genotype incompatibility, but not for male offspring.

A second area of research would focus on if/how the maternal-fetal genotype incompatibility integrates with genetic liability for schizophrenia (phenocopy, gene-environment covariation, and gene-environment interaction). The phenocopy and gene-environment covariation models are unlikely to explain the association between *RHD* maternal-fetal genotype incompatibility and schizophrenia. However, one possible explanation for the finding that most people with a history of Rhesus D incompatibility do not develop schizophrenia is that the schizophrenia-producing effect of *RHD* maternal-fetal genotype incompatibility manifests only in individuals with genetic predisposition to schizophrenia.

This is a gene-"environment" interaction hypothesis. If this is the case, then one would expect different risks for schizophrenia based on family history and *RHD* maternal-fetal genotype incompatibility, with greatest risk of schizophrenia among genetically high risk individuals who are exposed to *RHD* maternal-fetal genotype incompatibility. Following the recent work of Clarke et al. [27], one could test for synergism between *RHD* maternal-fetal genotype incompatibility and family history of psychosis by comparing the rates of schizophrenia across four groups: no *RHD* maternal-fetal genotype incompatibility and no family history of psychosis, *RHD* maternal-fetal genotype only, family history of psychosis only, and *RHD* maternal-fetal genotype incompatibility and positive family history of psychosis. The same interaction hypothesis could be tested for *HLA-B* maternal-fetal genotype incompatibility; however additional research is needed also to test the phenocopy and gene-environment covariation models with respect to the association between *HLA-B* matching and schizophrenia.

A third area of research would focus on hypotheses that hypoxia is the prominent schizophrenia-producing effect of *RHD*/*HLA-B* maternal-fetal genotype incompatibility. As examples, if the schizophrenia risk effect of *RHD* maternal-fetal genotype incompatibility is the result of hypoxia, then one would expect to observe an interaction between *RHD* maternal-fetal genotype incompatibility and hypoxia-regulated/vascular-expression genes. The same hypothesis can be tested with *HLA-B* maternal-fetal genotype incompatibility. If the schizophrenia risk effect of *RHD* maternal-fetal genotype incompatibility is the result of hypoxia, then one would expect to observe smaller hippocampal volume in individuals with schizophrenia exposed to *RHD* maternal-fetal genotype incompatibility compared to those not exposed. The same hypothesis can be tested with *HLA-B* maternal-fetal genotype incompatibility. If the schizophrenia risk effect of *RHD* maternal-fetal genotype incompatibility is the result of hypoxia, then one would expect *RHD* maternal-fetal genotype incompatibility to be associated with neurocognitive functions that may be sensitive to the effects of prenatal hypoxia in schizophrenia, for example, verbal learning and memory. The same hypothesis can be tested with *HLA-B* maternal-fetal genotype incompatibility.

A fourth area of research would focus on hypotheses to further examine offspring sex-dependent differences in the schizophrenia-producing effects of *RHD* maternal-fetal genotype incompatibility. For this area of research, hypotheses regarding sex-dependent differences in amount of exposure (threshold effect), gestational timing of exposure (timing effect), and type of exposure, that is, hypoxia and hyperbilirubinemia (specific effect) are likely best tested using animal models which can systematically vary conditions of hypoxia and hyperbilirubinemia. Similar investigations can be performed when the prenatal effects of *HLA-B* maternal-fetal genotype incompatibility are better elucidated.

A fifth area of research would focus on identifying other "incompatibility" genes. The attributable risk associated with these maternal-fetal genotype incompatibilities is limited to populations in which the incompatibility occurs with appreciable frequency. As one example, the Rhesus D negative allele is less common in African and Asian populations than European Caucasian populations [72, 120, 121], hence *RHD* maternal-fetal genotype incompatibility is less likely to contribute to schizophrenia susceptibility in those populations. However, *RHD* is not the only blood antigen locus for which a maternal-fetal genotype incompatibility could arise. Other blood antigens exist, including *ABO* [48], *RHCE* [122], Kell [123, 124], Duffy [125], Kidd [48], and MN [125, 126] and maternal-fetal genotype incompatibilities for these antigens can give rise to a maternal immune response that is similar, although smaller in magnitude, to the *RHD* incompatibility response. In addition, other genes that could lead to fetal hypoxia, hyperbilirubinemia, or other prenatal conditions associated with schizophrenia, whether through maternal-fetal genotype incompatibility, maternal genetic effects alone, or fetal genetic effects alone, should be examined.

5. Conclusion

Prenatal environmental factors are quite heterogeneous and difficult to document reliably, making it difficult to test the role of environmental insults in the pathogenesis of schizophrenia. However, there is growing evidence that many prenatal/obstetric complications have a genetic basis, and one stream of research has focused on identifying combinations of maternal-fetal genotypes, that is, maternal-fetal genotype incompatibilities, that predispose to prenatal/obstetric complications. Maternal-fetal genotype incompatibility can occur when maternal and fetal genotypes differ, for example, *RHD* maternal-fetal genotype incompatibility, or when they are too similar, for example, *HLA-B* maternal-fetal genotype incompatibility. Thus far, the *RHD*, *ABO*, and *HLA-B* genes have been implicated as risk factors for schizophrenia, with increasing evidence that male and female offspring may be differentially vulnerable to the effects of maternal-fetal genotype combinations involving these genes. A growing number of studies demonstrate that an interaction between prenatal/obstetric complications and putative susceptibility genes increases risk for schizophrenia. Thus, these maternal-fetal genotype incompatibilities are likely to be part of a complex mixture of factors (genetic and environmental), which together act on the brain in ways yet to be identified to result in schizophrenia. The empiric data demonstrating a relationship between these maternal-fetal genotype incompatibilities and schizophrenia provide hypotheses for future investigations to further our understanding of their role in increasing risk of schizophrenia.

Until recently, studies to understand the role of maternal-fetal genotype incompatibility in schizophrenia (or any complex disorder) have inferred immunologically relevant genotypes solely from birth records and for the single phenomenon of hemolytic disease. As illustrated in this review, maternal-fetal genotype incompatibility at other loci, such as *HLA* loci, may also increase risk for schizophrenia. However, because these loci do not result in hemolytic disease of the newborn it may be challenging, a priori, to examine their role through information gleaned from birth records. Hence, the development of study designs and statistical methods to study prenatal risk factors based on

genotype data are essential for further delineating maternal-fetal genotype incompatibility as a non-Mendelian mechanism in complex disease. In fact, genetic studies that do not model non-Mendelian patterns of inheritance directly may be one contributing reason that current genome scans have not found striking and highly replicable results in complex disorders that otherwise are so highly familial.

The approach described here integrates the investigation of genes and environment in an innovative manner and provides empirical data that fits within and can be further tested in a genetic-inflammatory-vascular hypothesis of schizophrenia. There are several reasons why it is important to further investigate maternal-fetal genotype incompatibly as a risk factor for schizophrenia: (1) it is a new research approach that allows precise identification of a putative high-risk prenatal environment, even years after the adverse environment has occurred; (2) using a genetic approach, it is possible to simultaneously evaluate alternative explanations of allelic effects that act solely through the genotype of the mother or child; (3) if certain maternal-fetal genotype incompatibilities, for example, *RHD*, do increase risk for schizophrenia, then efforts could be launched to increase prevention of the effects of this class of risk factor; (4) this approach could serve as a model for studying other complex disorders for which maternal-fetal genotype incompatibilities may be involved, for example, diabetes [127, 128] and rheumatoid arthritis [129–131].

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Research Article

The Relationship between Birthweight and Longitudinal Changes of Blood Pressure Is Modulated by Beta-Adrenergic Receptor Genes: The Bogalusa Heart Study

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This study examines the genetic influence of β-adrenergic receptor gene polymorphisms (β_2-AR Arg16Gly and β_3-AR Trp64Arg) on the relationship of birthweight to longitudinal changes of blood pressure (BP) from childhood to adulthood in 224 black and 515 white adults, aged 21–47 years, enrolled in the Bogalusa Heart Study. Blacks showed significantly lower birthweight and frequencies of β_2-AR Gly16 and β_3-AR Trp64 alleles and higher BP levels and age-related trends than whites. In multivariable regression analyses using race-adjusted BP and birthweight, low birthweight was associated with greater increase in age-related trend of systolic BP (standardized regression coefficient $\beta = -0.09$, $P = .002$) and diastolic BP ($\beta = -0.07$, $P = .037$) in the combined sample of blacks and whites, adjusting for the first BP measurement in childhood, sex, age, and gestational age. Adjustment for the current body mass index strengthened the birthweight-BP association. Importantly, the strength of the association, measured as regression coefficients, was modulated by the combination of β_2-AR and β_3-AR genotypes for systolic ($P = .042$ for interaction) and diastolic BP age-related trend ($P = .039$ for interaction), with blacks and whites showing a similar trend in the interaction. These findings indicate that the intrauterine programming of BP regulation later in life depends on β-AR genotypes.

1. Introduction

Low birthweight is an indicator of intrauterine growth retardation. The relationship between low birthweight and elevated blood pressure (BP) levels later in life and the mechanisms of fetal programming have been extensively studied since the fetal origins hypothesis was proposed [1–4]. However, evidence for the developmental programming of adult hypertension thus far is still inconsistent, particularly in pediatric populations [5–7]. Recently, researchers have outlined substantive challenges to the birthweight-BP association. One of the criticisms is the inappropriate use of statistical methodology and improper interpretation of epidemiologic analyses in support of the fetal origins hypothesis [3, 8–12].

β-adrenergic receptors (β-ARs) play a pivotal role in regulation of cardiac, pulmonary, vascular, endocrine, and sympathetic nervous systems [13, 14]. Three β-AR subtypes (β_1-AR, β_2-AR, and β_3-AR) are pharmaceutically characterized [14, 15]. Polymorphisms of the β-AR genes have been found to be associated with multiple cardiovascular risk variables, including hemodynamic factors such as heart rate, vasodilation, and BP [16]. Further, the β-AR genes are associated with preterm birth and modulate the relationship between birthweight and insulin resistance in later life [17–20]. However, whether genetic variations in the β-AR genes modulate the influence of birthweight on BP is not known, especially for BP longitudinal changes.

As part of the Bogalusa Heart Study, a long-term biracial (black-white) community-based epidemiologic study of the early natural history of cardiovascular disease beginning in childhood since 1973 [21], the present study examines the modulating effect of β_2-AR Arg16Gly and β_3-AR Trp64Arg polymorphisms on the association between birthweight and

longitudinal changes of BP from childhood to adulthood in black and white asymptomatic younger adults enrolled in the Bogalusa Heart Study.

2. Methods

2.1. Study Cohort. In the community of Bogalusa, LA, 9 cross-sectional surveys of children aged 4–17 years between 1973 and 1994 and 10 cross-sectional surveys of young adults aged 18–48 years between 1987 and 2009, who had been previously examined as children, were conducted for cardiovascular risk factors. This panel design of repeated cross-sectional examinations conducted approximately every 3 years has resulted in serial observations from childhood to adulthood. A total of 739 adult subjects (515 whites and 224 blacks; 42.2% males; age range = 21–47 years; mean age = 36.7 years) who had β-AR gene polymorphism genotype data formed the study cohort for this report. The study subjects have been serially examined 4–14 times (at least 2 times each in childhood and adulthood) for BP over an average of 26.7 years, with 5793 observations. Birthweight records of the participants were obtained from the Office of Health Statistics in New Orleans, Louisiana. All subjects in this study gave informed consent at each examination, and for those under 18 years of age, consent of a parent/guardian was obtained. Study protocols were approved by the Institutional Review Board of the Tulane University Health Sciences Center.

2.2. General Examinations. All surveys since 1973 followed the same protocols, and procedures for the general examinations were described elsewhere [22]. Height and weight were measured twice to ± 0.1 cm and to ± 0.1 kg, respectively. Body mass index (BMI, weight in kilograms divided by the square of the height in meters) was used as a measure of overall adiposity. BP levels were measured on the right arm of subjects in a relaxed, sitting position by 2 nurses (3 replicates each). The first and fourth Korotkoff phases were used to determine systolic (SBP) and diastolic (DBP) blood pressures, respectively. Means of replicate readings were used for analyses. For subjects ($n = 57$) who were on medications for hypertension at the time of examination, we adjusted the recorded BP levels by adding 10 mm Hg to SBP and 5 mm Hg to DBP, based on average treatment effects [23, 24].

2.3. Genotyping. Genotyping of β_2-AR Arg16Gly and β_3-AR Trp64Arg polymorphisms was performed using the TaqMan assay (Applied Biosystems, Inc., Foster, CA) in the laboratory of the Human Genetics Center, University of Texas School of Public Health, 1200 Hermann Pressler, E447, Houston. Amplification conditions and information on primer and probe sequences have been previously described [25, 26]. The reproducibility of genotyping of the three polymorphisms was 100% assessed by 64 blind duplicates.

2.4. Statistical Methods. Cubic growth curves of BP measured repeatedly at multiple time points from childhood to adulthood were established by race and sex groups using a random effects model by SAS Proc MIXED. As shown in Figure 1 using SBP of two white males as an example, the area under the curve (AUC) was calculated as the integral of the growth curve parameters during the follow-up period for each individual [27, 28]. Total AUC (baseline AUC + incremental AUC) is considered a measure of long-term levels. Incremental AUC determined by within-individual variability represents a combination of linear and nonlinear longitudinal trends and was used as the measure of age-related trend. Since individuals had different follow-up periods, the AUC values were divided by the number of follow-up years for further analyses. The AUC measures have advantages over other longitudinal analysis models in that they measure both the long-term levels and trends.

Race differences in distributions of genotypes and allele frequencies were tested using a contingency chi-square test. Differences in mean values of continuous study variables between race-sex groups were tested by analysis of covariance models. The rate of fetal growth was calculated as birthweight in kilograms/gestational age in weeks for each individual, and then multiplied by the mean gestational age of the sample to make the scale close to the original values. The gestational age-adjusted birthweight was used in all subsequent analyses. The impact of genotype and birthweight on BP was examined by multiple regression models by race and in the total sample, adjusting for sex, current age, and race (for the total sample). In order to examine the impact of BMI adjustment on the relation between birthweight and BP, two types of regression analyses were performed, with and without adjustment for current BMI. In addition to the above covariates, the baseline BP (the first measurement in childhood) was also included in the regression models for the age-related trend analyses to control for the regression-to-the-mean bias because the baseline values were closely associated with the age-related trend measured by incremental AUC. The differences in slopes (birthweight-genotype interaction on BP) were tested using a homogeneity-of-slopes model, a regression interaction model, among genotype groups. Due to significant differences in BP levels, birthweight and gene allele frequencies between blacks and whites, BP measures (childhood, adulthood, AUC, and age-related trend), and birthweight were standardized into Z-scores (mean = 0 and SD = 1) by race groups prior to regression analyses to remove the influence of population stratification.

3. Results

Table 1 shows the mean levels of study variables in childhood, adulthood, and at birth, and AUC values by race and sex. The first measurement of childhood SBP and DBP did not significantly differ between races or sexes. The last measurement of adulthood SBP and DBP differed significantly by race (blacks > whites) and by sex (males > females) except for sex difference in blacks. Total AUC and age-related trend of SBP showed significant race (blacks > whites) and sex (males > females) differences; significant sex differences (males > females) were noted for total DBP AUC in both races and a significant race difference (blacks > whites) in females only. The age-related trend of DBP showed

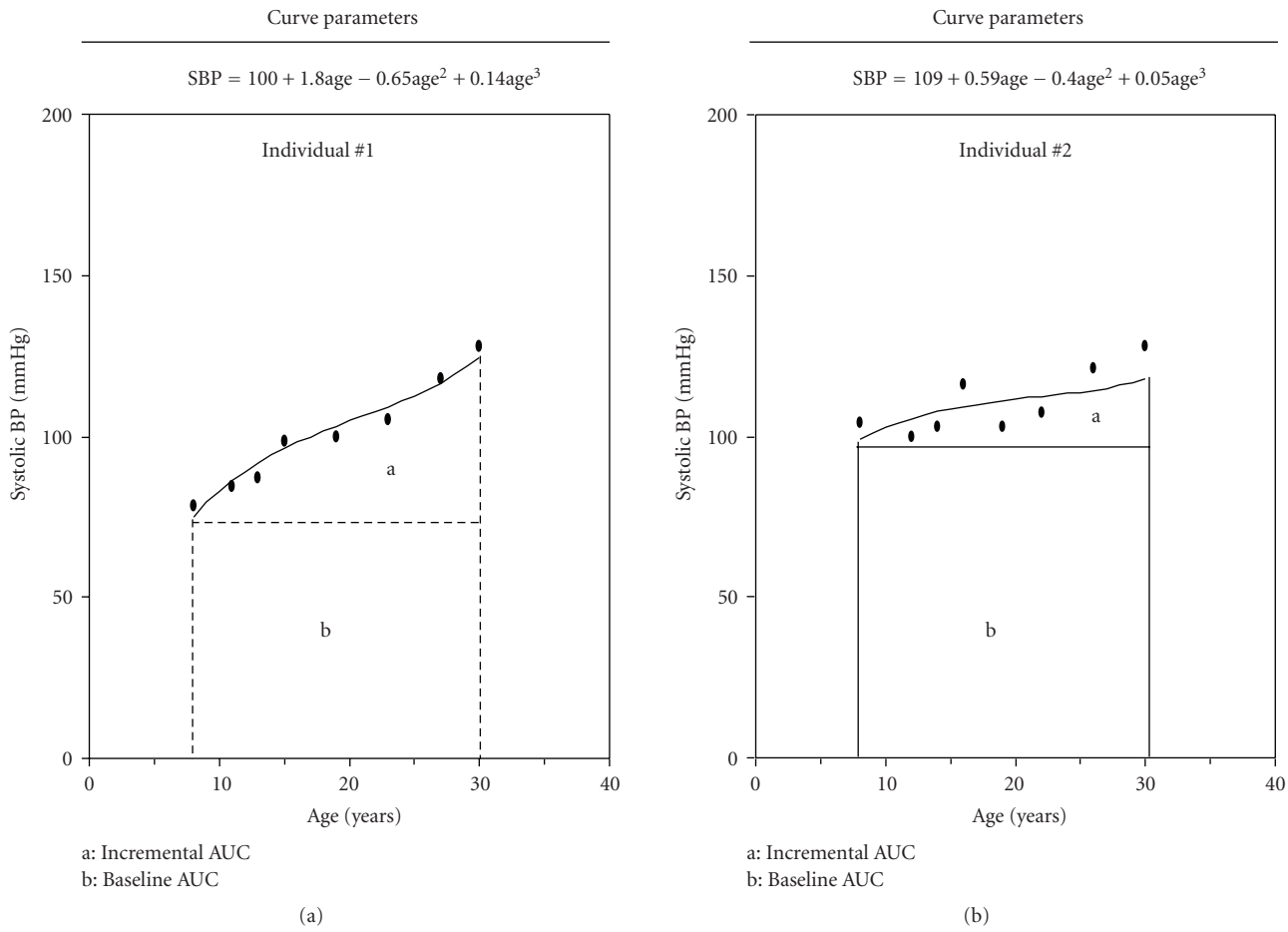

Figure 1: Illustration for Calculation of Area under the Curve of Systolic Blood Pressure: The Bogalusa Heart Study.

a significant race difference (blacks > whites) among females only and a sex difference (males > females) among whites only. Blacks had significantly lower birthweight than whites, but males had significantly higher birth weight than females only among whites.

Blacks displayed a lower alleles frequencies of β_2-AR Gly16 (0.507 versus 0.630, $P < .001$) and β_3-AR Trp64 (0.895 versus 0.925, $P = .008$) than whites. The genotype distributions were in accordance with Hardy-Weinberg equilibrium expectations in blacks and whites (data not shown).

Standardized regression coefficients for β-AR genotypes, race-adjusted birthweight, and covariates for race-adjusted BP are presented in two models in Table 2. Since race-birthweight and race-gene interactions on BP were not significant, the results of the total sample with blacks and whites combined are displayed. Race was not included in the models because birthweight and BP were adjusted for race by standardization prior to the regression analyses. The associations between race and Z-scores of BP measures (childhood, adulthood, AUC and age-related trend) were no longer significant ($P = .725$–$.896$). In model I with current BMI included, childhood age and current BMI, but not birthweight, were associated with the first SBP measurement in childhood. Female sex, older age, higher adulthood BMI, and lower birthweight were significantly associated with higher values of the last SBP measurement in adulthood, total SBP AUC, and SBP age-related trend. Birthweight was significantly associated with adulthood DBP levels and age-related trend; however, the association was weaker than for SBP. In addition, the first BP measurement in childhood (the baseline value) was also a significant predictor of the age-related trend of both SBP and DBP. In model II without current BMI included, the effect of birthweight on SBP was attenuated to some extent for adulthood SBP, SBP AUC, and the age-related trend, but they were still significant; the regression coefficients for other covariates did not considerably changed compared with those in model I. Birthweight was associated with the age-related trend only for DBP in model II. The main effect of individual β2-AR and β3-AR polymorphism genotype on SBP and DBP was not significant in both model I and model II without any interaction terms included.

Figure 2 illustrates the magnitude of the association of birthweight with SBP age-related trend, measured as standardized regression coefficients, by the combination of β_2-AR and β_3-AR genotypes. The regression coefficients of SBP age-related trend on birthweight were derived from multiple regression models using race-adjusted birthweight and BP

Table 1: Mean Levels and SD of Study Variables by Race and Sex.

	Whites				Blacks					
	Males ($n = 226$)		Females ($n = 289$)		Males ($n = 86$)		Females ($n = 138$)		P-value for difference	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Race	Sex
Childhood										
Age (year)	10.3	2.9	9.7	3.0	10.0	2.9	9.6	2.7	NS	<.05 w
Systolic BP (mm Hg)	100.8	9.4	99.4	9.1	99.8	11.8	99.0	9.6	NS	NS
Diastolic BP (mm Hg)	61.7	8.2	62.2	8.8	62.4	8.0	61.0	8.6	NS	NS
Adulthood										
Age (year)	37.6	5.1	36.7	5.5	35.0	5.9	35.9	5.7	<.01 m	NS
Systolic BP (mm Hg)	117.1	10.7	109.5	10.8	122.8	15.2	119.3	17.3	<.01	<.05 w
Diastolic BP (mm Hg)	78.9	8.0	73.8	7.9	81.2	11.8	78.7	11.5	<.05	<.05
AUC Values (mm Hg)										
Systolic BP total	113.1	7.2	106.9	5.5	115.0	7.6	110.7	6.8	<.05	<.01
Systolic BP trend	11.8	5.1	7.5	5.3	15.6	6.6	11.8	6.2	<.01	<.01
Diastolic BP total	72.3	5.8	69.7	4.5	72.3	6.2	70.8	5.0	<.01 f	<.05
Diastolic BP trend	10.8	3.3	8.1	4.0	10.8	3.8	9.7	4.2	<.01 f	<.01 w
At Birth										
Gestational age (week)	39.8	1.6	39.6	2.0	39.2	2.3	39.5	1.8	<.05 m	NS
Birth weight (kg)	3.46	0.5	3.34	0.6	3.09	0.6	3.00	0.6	<.01	<.05 w
Birth weight (kg)†	3.44	0.5	3.33	0.5	3.10	0.5	3.00	0.5	<.01	<.05 w

BP: blood pressure; AUC: area under the curve.
NS : $P > .05$; f : females only; m: males only; w : whites only.
† adjusted for gestational age.

Table 2: Standardized regression coefficients of race-adjusted blood pressure on birth weight, β-adrenergic receptor genotypes, and covariates.

	Systolic BP				Diastolic BP			
	First	Last	AUC	Trend	First	Last	AUC	Trend
Model I								
Childhood BP	—	—	—	−0.43**	—	—	—	−0.44**
Female sex	−0.05	−0.24**	−0.37**	−0.38**	0.02	−0.22**	−0.19**	−0.28**
Age	0.22**	0.19**	0.17**	−0.20**	0.28**	0.25**	0.34**	−0.02
BMI	0.16**	0.36**	0.26**	0.23**	0.06	0.34**	0.21**	0.26**
Birth weight†	−0.004	−0.11**	−0.11**	−0.10**	0.04	−0.07*	−0.07*	−0.08*
β_2-AR genotype	−0.04	−0.03	−0.04	0.01	−0.05	−0.02	−0.03	−0.01
β_3-AR genotype	−0.04	−0.01	−0.02	0.02	−0.05	−0.01	−0.05	−0.01
Model II								
Childhood BP	—	—	—	−0.40**	—	—	—	−0.42**
Female sex	−0.05	−0.25**	−0.38**	−0.39**	0.01	−0.24**	−0.20**	−0.29**
Age	0.23**	0.22**	0.19**	−0.19**	0.28**	0.28**	0.36**	−0.01
Birth weight†	−0.004	−0.09**	−0.10**	−0.09**	0.04	−0.05	−0.06	−0.07*
β_2-AR genotype	−0.05	−0.05	−0.05	−0.02	−0.05	−0.04	−0.04	−0.02
β_3-AR genotype	−0.04	−0.01	−0.02	0.02	−0.05	−0.01	−0.05	−0.01

First: the first measurement in childhood; last: the last measurement in adulthood; BP: blood pressure;
AUC: area under the curve; BMI: body mass index.
β_2-AR genotype = β_2-adrenergic receptor Arg16Gly (coding: 0, 1, and 2 of Arg alleles).
β_3-AR genotype = β_3- adrenergic receptor Trp64Arg (coding: 0 = Arg/Arg and Trp/Arg, 1 = Trp/Trp).
† adjusted for gestational age.
*$P < .05$; **$P < .01$.

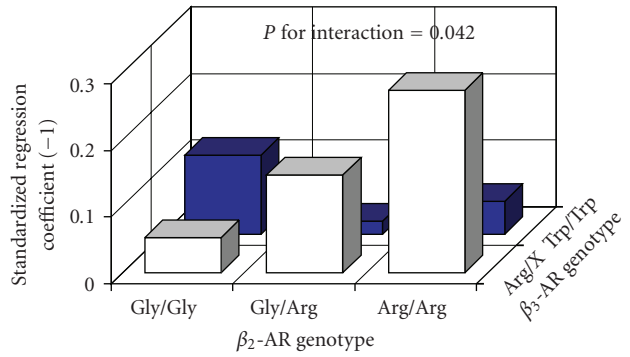

Figure 2: Relationship between Age-related Trend of Systolic Blood Pressure and Birth Weight by β_2-AR Arg16Gly and β_3-AR Trp64Arg Genotypes: The Bogalusa Heart Study Arg/x stands for Arg/Trp and Arg/Arg genotypes combined.

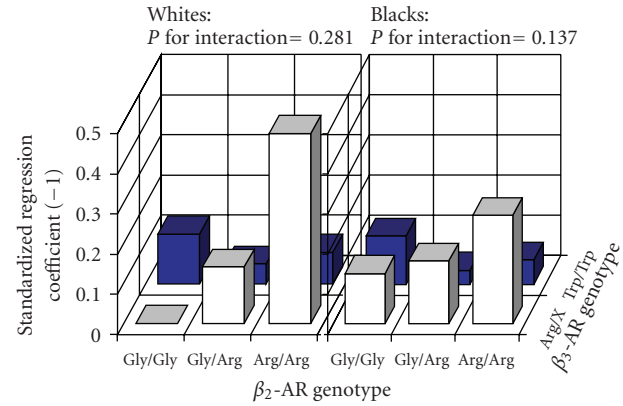

Figure 3: Relationship between Age-related Trend of Systolic Blood Pressure and Birth Weight by β_2-AR Arg16Gly and β_3-AR Trp64Arg Genotypes in Blacks and Whites: The Bogalusa Heart Study Arg/x stands for Arg/Trp and Arg/Arg genotypes combined.

in terms of race-specific Z-scores, adjusting for the first SBP measurement in childhood, adulthood age, and sex, without any interaction terms. Because the regression coefficients were all negative, standardized regression coefficients $\times(-1)$ were presented in Figure 2 for convenience. The regression coefficients showed opposite trends with increasing number of the β_2-AR Arg16 alleles in the two β_3-AR genotype groups. The birthweight effect on SBP trend ($\beta = -0.12$, $P = .014$, $n = 224$) was the greatest among individuals who had β_3-AR Trp/Trp and β_2-AR Gly/Gly genotypes; having β_3-AR Arg/x and β_2-AR Arg/Arg genotypes was associated with the highest effect of $\beta = -0.28$ ($P = .257$, $n = 21$). The overall 3-way interaction (birthweight-β_2-AR-β_3-AR) was significant (P = .042), suggesting that the relationship between SBP age-related trend and birthweight depends on the combination of β_2-AR and β_3-AR genotypes. Further, the interaction of individual polymorphisms with birthweight on SBP trend was not significant in the 2-way interaction models without a 3-way interaction term included (data not shown).

Figure 3 presents the strength of the association of birthweight with SBP age-related trend by the combination of β_2-AR and β_3-AR genotypes in blacks and whites using the race-specific Z-scores of birthweight and SBP age-related trend. The pattern of the standardized regression coefficients of SBP age-related trend on birthweight by β_2-AR and β_3-AR genotypes was similar in the two race groups; the four-way interaction (race-birthweight-β_2-AR-β_3-AR) was not significant ($P = .523$).

For DBP age-related trend, a similar pattern of regression coefficients to SBP was observed with respect to β_2-AR and β_3-AR genotypes; the birthweight-β_2-AR-β_3-AR interaction on DBP age-related trend was significant ($P = .039$) in the total sample. Blacks and whites did not show any difference in the birthweight-genotype interaction on DBP age-related trend ($P = .682$).

4. Discussion

Many studies have shown that low birthweight is inversely associated with high BP and hypertension in later life [1–4] although there exist criticisms and concerns [3, 8–12]. Systematic reviews of the literature suggest an inverse linear relationship between SBP and birthweight of ~1-2 mm Hg/kg [29]. However, most previous studies in this regard focused on BP levels; limited information is available on the influence of birthweight on longitudinal changes of BP. The present community-based study demonstrates an adverse effect of low birthweight on BP in terms of adulthood BP, the long-term levels, and increasing trend from childhood to young adulthood, especially for SBP. However, the birthweight-BP association was not noted in childhood in the study cohort.

The hypothesis of the fetal origins of hypertension proposed several possible mechanisms linking reduced fetal growth and elevated BP in later life, such as persisting changes in vascular structure, including loss of elasticity in vessel walls, effects of glucocorticoid hormones, and abnormalities of nephrogenesis [1, 2]. Furthermore, low birthweight may be associated with elevated sympathetic nervous system activity in adulthood, as measured by heart rate, preejection period, and respiratory sinus arrhythmia [30, 31]. These findings suggest that increased sympathetic nervous system activity established in utero may be one of the mechanisms linking low birthweight with high BP in adults. Although genetic factors have been found to play a role in the birthweight-BP relationship in family and twin studies [31–33], limited information on specific genes has become available. We noted in the current study that the association between birthweight and age-related trend of BP was dependent on the combination of by β_2-AR and β_3-AR genotypes, suggesting that β-AR gene variants play a role, in an interactive manner, in BP regulation of adults who have a low birthweight, an indicator of fetal growth restriction.

It is well established that both BP regulation and birthweight are genetically determined [34, 35]. Candidate genes and chromosomal regions have been identified for birthweight [36, 37]. β-ARs are an important component of the sympathetic nervous system [13, 14]. Polymorphisms of the β-AR genes have been found to be associated with

multiple cardiovascular risk factors, including hemodynamic status and obesity measures. Previous studies have shown that β_2-AR Gly16 and β_3-AR Arg64 alleles were associated with BP elevation or hypertension, although the findings on β_2-AR Gly16 allele have been questioned [16, 38]. In the present study, we did not find a significant association of β_2-AR Arg16Gly and β_3-AR Trp64Arg polymorphisms, individually, with BP in terms of childhood, adulthood, and long-term BP levels, and the age-related trend. However, the two genes were found to interactively influence the relationship of birthweight to longitudinal changes of BP. Individuals who were born a lower weight and have β_2-AR Arg/Arg and β_3-AR Arg allele or β_2-AR Gly/Gly and β_3-AR Trp/Trp genotypes are prone to have a faster increase in BP after birth compared with those having a normal birthweight and other β_2-AR and β_3-AR genotypes. Data on 3-way interaction (birthweight-β_2-AR-β_3-AR) on BP, which is a complex interplay, have not been previously reported. Further studies with multiple polymorphisms of the two genes are needed to confirm the findings in this study with respect to the possible important loci which are in linkage disequilibrium with β_2-AR Arg16Gly and β_3-AR Trp64Arg polymorphisms.

In the United States, blacks have a greater prevalence of both low birthweight and hypertension than whites; however, it is not yet established that excess risk of hypertension in blacks is programmed in utero [39]. In some studies black children showed a weaker birthweight-BP association than white children, or none at all [40–43]. A weaker birthweight-BP association in blacks was also observed in our previous study in a separate cohort [44]. However, the modulating effect of β_2-AR and β_3-AR genes, individually or in combination, on the association between birthweight and longitudinal changes of BP did not differ between blacks and whites in the present study. In addition to race differences in brithweight and BP, β_2-AR and β_3-AR genes allele frequencies also differed significantly between blacks and whites in this study cohort. Strong correlations among these study variables would lead to a serious bias resulted from population stratification when blacks and whites were combined for analysis. For this reason, BP and birthweight were standardized by race groups, and then race-specific Z-scores were used in association and interaction analyses in the present study.

It is well known that size at birth is strongly related to fetal genotype, uterine environment, and a number of maternal factors including parity, length of gestation, mother's adult size, mother's own birthweight, as well as mother's genotype [34, 45]. Because of the correlation of maternal and fetal genotypes of multiple relevant genes, maternal genotypes are expected to have a confounding effect on the birthweight-BP relationship as well as its genetic modulation by β_2-AR and β_3-AR gene polymorphisms noted in this study. However, mother's genotype data are not available in our cohort. Another limitation of this community-based study is that only one single polymorphism was typed in each of the two genes under study.

In summary, we found in the current study that low birth weight was associated with a greater increase in BP levels from childhood to adulthood. This relationship was modulated by the combination of β2-AR and β3-AR genotypes. These findings support the emerging concept of intrauterine programming and its pathophysiologic consequences after birth and underscore the role of genetic factors in the fetal origins of hypertension later in life. Since no data in this regard are available for comparison, our findings need to be replicated in other populations.

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Review Article

Genetic Risk for Recurrent Urinary Tract Infections in Humans: A Systematic Review

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Urinary tract infections (UTIs) are a frequent cause of morbidity in children and adults and affect up to 10% of children; its recurrence rate is estimated at 30–40%. UTI may occur in up to 50% of all women in their lifetimes and frequently require medication. Recent advances have suggested that a deregulation of candidate genes in humans may predispose patients to recurrent UTI. The identification of a genetic component of UTI recurrences will make it possible to diagnose at-risk adults and to predict genetic recurrences in their offspring. Six out of 14 genes investigated in humans may be associated with susceptibility to recurrent UTI in humans. In particular, the HSPA1B, CXCR1 & 2, TLR2, TLR4, TGF-β1 genes seem to be associated with an alteration of the host response to UTIs at various levels.

1. Background

Urinary tract infections (UTIs) are a frequent cause of morbidity in children and adults. UTIs affect up to 10% of the pediatric population [1] and the recurrence rate of UTI in children is estimated at 30% to 40%, with the majority of recurrences occurring in the first 12 months after the initial infection [2, 3]. Recurrent UTI is more frequent in very young females older than 6 months [4]. UTIs may occur in up to 50% of all women in their lifetimes and 10%–15% of cases require medication [5]. Susceptibility to recurrent UTIs in women could be the result of a combination of various risk factors such as periurethral bacterial colonization and Escherichia coli virulence [6].

The pathogenesis of recurrent UTIs consists of the three classic components of any infection: host, pathogen and environment [6]. The severity of a UTIs is determined by the innate defense mechanisms of the host and by the virulence of the infecting agents. The pathogenesis of the inflammation involves the recruitment of neutrophils into the urinary tracts and chemokines-chemokine receptor interactions. An inhibition of mucosal signaling creates a state of asymptomatic bacterial carriage (asymptomatic bacteriuria). Intricate molecular interactions govern pathogen recognition on mucosal surfaces as well as bacterial virulence. The innate host defense mechanisms determine the severity of infection [7].

For unknown reasons, UTIs are clustered in certain individuals. UTIs are more prevalent in female relatives of women with recurrent UTIs, which suggests a familial genetic predisposition to the disease [8–10]. Animal models have shown the multigenic nature of recurrent UTIs. Mouse models of induced, unobstructed UTI have been important in showing the influence of genetic factors on host susceptibility and resistance to bladder and kidney infections [11, 12]. Other studies on both experimental mice and humans

have disclosed that a predisposition to UTIrecurrences could be inherited, suggesting that the increased susceptibility to UTI is a complex heritable trait influenced by several genes [13]. Putative candidate genes for the disease include genes that are involved in the antimicrobial defense of surface epithelia.

The objectives of this systematic review were to present the genetic knowledge on the susceptibility to UTI recurrences in humans.

2. Methods

Using electronic databases, we looked for suitable comparative studies on children and adults that investigated the genetic susceptibility to recurrent UTIs. Participants were individuals of either gender with known or unknown familial history of UTIs. UTIs occurred at both high (pyelonephritis) or low (cystitis) urinary tract levels. We excluded studies on animals. Clinical investigations for UTIs included microbiological urinalysis and instrumental investigations (ultrasound, voiding cystoureterogram and DMSA scintigraphy). We excluded studies enrolling patients with urinary tract obstruction. The included studies were aimed at obtaining allele and genotype frequencies of gene polymorphisms/mutations and gene expression of specific loci recognized/suspected to be involved in UTIs recurrences in both experimental patients and controls.

We searched the MEDLINE, SCOPUS and EMBASE sites from 1985 through July 2009. We did not research literature prior to 1985 because genes relating to UTIs recurrences only began to be investigated in animals from 1985 [14, 15]. The first study that suspected a genetic predisposition to UTI recurrences in both mice and humans was published in 2000 [16]. The MeSH terms and text words (and their combinations and truncated synonyms) were adapted as appropriate to search each database by combining the terms "urinary tract infection AND (genetic predisposition OR genetic susceptibility OR genetic variation OR genetic mutation OR genetic polymorphism) AND human." We also did a manual search of all the bibliographies from the selected studies and from relevant review articles.

In some of the selected studies, we retrieved the genotype frequency distribution for each gene polymorphism investigated in patients with recurrent UTIs and controls. We retrieved the odds ratio (OR) and the confidence interval (95% CI) and/or the results of the statistical analyses (P values) that compared the frequency of gene polymorphisms between cases and controls. Moreover, we retrieved information on mRNA levels and the protein expression of candidate genes.

To our knowledge, no genome-wide association study has been done on a large sample of individuals to discover novel genetic caused susceptibility factors for UTIs. To date, association with UTI has been studied from a candidate gene approach only. The association between genetic variability and clinical susceptibility to disease often lacks definitive evidence due to methodological issues, high heterogeneity and deaths which preclude meta-analytical association studies [25]. It is also worth noting that the significant genetic associations reported could also include false positive results [26]. Thus, we investigated the methodological quality of each enrolled study. In particular, we looked to see if both genetic and protein expressions were investigated in the same study, if the ethnicity of the target population was reported, if the Hardy-Weinberg equilibrium was ascertained in both the case and control groups, if the authors declared any UTIs recurrence and the presence or not of a urinary tract malformation in their patients.

3. Results

Our initial search into the literature identified a large number of papers from PUBMED (236 found), SCOPUS (680) and EMBASE (80). After a hand screening for titles and abstracts, 21 studies were considered to be relevant to this review. A closer look at these 21 papers excluded 11 papers because they investigated the genetic susceptibility to renal damage after aUTI [27–37]. Finally, ten studies were identified from the literature as being eligible for this review [16–25]. Among them, two studies referred to the same protocol and patients [13, 16]. Seven studies investigated the frequency of target gene polymorphisms in patients with recurrent UTIs [17, 18, 20–24], one of which lacked a control group [20]. Moreover, five studies investigated the expression of mRNA or the proteins of candidate genes associated with UTI recurrence [16, 18–20, 23]. In summary, the nine studies we settled on included only an assessment of gene polymorphisms [17, 21, 22, 24], only mRNA or protein expression assessment [13, 16, 19] or both [18, 20, 23].

3.1. IL-8 Receptor CXCR1 and CXCR2 Genes. Frendéus et al. investigated the neutrophil CXCR1 and CXCR2 surface expressions in mice and humans. Twelve children with recurrent UTIs showed decreased expression of CXCR1 protein and mRNA levels in neutrophils of children prone to pyelonephritis [16]. CXCR2 expression was comparable between patients and controls [13]. Smithson et al. investigated the surface expression of CXCR1 and CXCR2 as well as the presence of CXCR1 gene polymorphisms in 20 premenopausal women with recurrent UTIs and normal urinary tracts and 30 females with no history of UTIs. CXCR1 expression was similar to that of healthy controls, whereas CXCR2 expression was lower than in controls. Sequence analysis of promoter and coding sequence of the CXCR1 gene showed the presence of three polymorphisms in the exon 2: one patient with R335C and two patients with S276T [20]. The study by Lundstedt et al. investigated the full-length CXCR1 gene sequence in 12 pediatric patients, 12 pediatric controls and 35 adults with childhood APN. The frequency of relevant CXCR1 gene polymorphisms was further assessed in 12 children, one adult, 14 control children, and 200 healthy adult blood donors. The novel 217C>G polymorphism of the CXCR1 gene was located in a putative binding site for the run-related transcript factor 1 (RUNX1), which is required for expression from

a number of cell specific enhancers and promoters. The allele 217G was observed to be associated with a reduction in RUNX1 binding. Moreover, aberrantly reduced CXCR1 mRNA processing from patients and mothers carrying the A allele of the 3665G>A polymorphism suggested that this variant might create a more efficient cleavage site, reducing the amount of large CXCR1 mRNA. Moreover, the CXCR1 expression by flow cytometry in 60 patients (24 children and 36 adults) with a history of childhood UTIs and controls (26 children and 200 adults) showed low CXCR1 surface neutrophil expression in patients prone to APN [18]. In another paper, the same authors investigated the CXCR1 surface expression in 10 children with a history of APN and recurrent UTI, as well as their families, and 15 controls. CXCR1 expression was lower in the patients and their families compared to controls [19].

3.2. Heat Shock Protein 72 (HSPA1B) Gene. Karoly et al. investigated the frequency of the 1267A>G polymorphism of the heat shock protein 72 gene (HSPA1B) and the frequency of the TLR4 896A>G polymorphism in 103 children with recurrent UTIs compared with 235 randomly selected controls. Clinical investigations identified vesicoureteral reflux (VUR) in 50 patients and renal scarring in 40 patients, more commonly in those suffering from high-grade VUR. The 1267G allele occurred more frequently in patients with recurrent UTIs [17].

3.3. Toll-like Receptor (TLR) Pathway Genes. The 896/AG genotype and the 896G allele of the TLR4 gene showed a higher prevalence among UTI patients than among controls. Moreover, the 896/AG genotype and 896G allele tended to occur more frequently in patients with recurrent UTIs without VUR [17].

Ragnarsdóttir et al. investigated the TLR4 protein expression in 17 children with asymptomatic bacteriuria (ABU) and 24 controls. They found reduced expression of the TLR4 protein. The authors recruited 41 pediatric and 29 adult patients and age-matched controls, to measure single immunoglobulin domain-containing IL- 1 receptor-related molecule (SIGIRR) and TLR4 adaptor protein TRIF expressions (TIR domain containing adaptor inducing interferon-β), TRAM (TRIF-related adaptor molecule) and MyD88 (Myeloid differentiation primary response gene 88). They found high levels of the TLR4 adaptor protein TRIF and reduced levels of the TLR4-inhibitor SIGIRR in the patients with ABU. They proceeded with DNA sequencing of TRL4 DNA in 28 children with ABU and 14 controls but they identified only one known missense mutation in one control subject. The TRIF gene was sequenced in 12 children and 11 controls. The frequency of TRIF polymorphisms was comparable between the patients with ABU and the control subjects [23].

Tabel et al. investigated the 2258G>A polymorphism (also described in the paper as G2251A or rs5743708 referring to Arg753Gln: http://www.snpedia.com/index.php/Rs5743708) of the TLR2 gene in 124 Turkish children with a first time or recurrent UTI. This study demonstrated a significantly higher frequency of the 2258A allele in the TLR2 gene in patients with recurrent UTIs than in 116 controls. The authors did not find any correlation between a TLR2 gene polymorphism and the localization of UTIs [22].

In a case-controlled designed study, Hawn et al. investigated the TLR pathway genes in 431 adult women with recurrent UTIs and 430 controls. Nine TLR pathway genes including TLR-1,2,4,5,6 and the genes of the TLR4 adaptor molecules MYD88, TIR domain containing adapter (TIRAP), TRIF, and TRAM, were sequenced at DNA level. In particular, 45 patients for the TLR2 gene, 43 for the TLR4 gene, 46 for the TLR5 gene, 86 for the TIRAP gene and 87 for the TRIF gene were investigated. The TLR5 1174C>T gene polymorphism was associated with increased susceptibility to recurrent cystitis. No significant difference in the frequency of the 2258A allele of the TLR2 gene was found between adult women with UTI, including urinary tract malformations (UTM), and controls. The TLR1 1805G>T and TLR4 896A>G polymorphisms were associated with protection from APN and recurrent cystitis, respectively [21].

3.4. VEGF and TGF-β1. Yim et al. investigated vascular endothelial growth factor (VEGF) and TGF-β1 gene polymorphisms with susceptibility to UTI. In particular, the authors investigated two polymorphisms of the VEGF gene (460T>C and 405G>C) and three polymorphisms of the TGF-β1 gene (-509C>T, -800G>A and 869T>C). The overall number of patients was 79, of which 48 patients had renal scars and 57 had VUR, and 89 were controls. An increased VEGF 460/CC, TGF-β1-509/CC, TGF-β1-800/GG genotype frequency and decreased VEGF 460/TC, TGF-β-509/TT, TGF-β1-800/GA genotype frequency were found in patients with UTI when compared with control groups, both including patients with VUR. Excluding patients with VUR, children with UTIs showed an increased frequency of TGF-β1 -509/CC genotype when compared to a control group [24].

3.5. Summary of the Results. Table 1 summarizes the main characteristics of the studies. Fourteen genes were investigated, in particular 10 genes for one or more polymorphisms, and 7 genes for their mRNA levels or protein expression. Only two studies including CXCR1 and TRIF genes combined the assessment of both polymorphisms and protein expression in patients with and without VUR [18, 23]. The majority of the studies included patients with VUR and the minority included different non-obstructive UTMs. Only two studies investigated CXCR1, CXCR2 protein expression and TLR2 polymorphism in patients without UTMs [20, 22]. Study populations included children only [13, 16, 17, 22, 24], both adults and children of either sex [18, 19, 23], or only adult women [21]. In a quote of the studies, the authors had not specified the ethnic group of the patients [13, 16, 19, 20, 23].

Table 2 describes the genetic investigation of patients with UTI susceptibility. The frequency of HSPA1B 1267A>G, CXCR1 217C>G and CXCR1 2608G>C gene allele was

Table 1: Summary of the characteristics of the included studies investigating the polymorphisms and/or mRNA or protein expression assessment in patients with recurrent urinary tract infections (CXCR: IL-8 receptor; HSPA1B: Heat shock protein 72; TGF: Transforming growth factor; TIRAP: TIR domain-containing adapter protein; TLR: Toll-like receptor; UTI: urinary tract infection; UTM: urinary tract malformation; VEGF: Vascular endothelial growth factor; VUR: vesicoureteral reflux).

Genes	Polymorphisms	Gene expression	Patient categories	Ethnicity (country)	Urological malformations included	References
HSPA1B	1267A>G	−	Children	Hungarian	VUR	[17]
CXCR1	—	+	Children	(Sweden)		[13, 16]
	217C>G	+	Children and adults	Caucasians	VUR	[18]
	2608G>C	+				
	—	+		(Sweden)		[19]
	—	+	Pre-menopausal women	(Spain)	—	[20]
CXCR2	—	+	Pre-menopausal women	(Spain)	—	[20]
	—	+	Children	(Sweden)	VUR	[13, 16]
TLR1	1805G>T	−	Adult women	Caucasian	UTM	[21]
TLR2	2258G>A	−	Children	Turkish	—	[22]
		−	Adult women	Caucasian	UTM	[21]
TLR4	896A>G	−	Adult women	Caucasian	UTM	[21]
	1196C>T	−				
	896A>G	−	Children	Hungarian	VUR	[17]
	—	+	Mainly children	(Sweden)		[23]
TRIF	9A>G	+	Mainly children	(Sweden)	VUR	[23]
TRAM	—	+	Mainly children	(Sweden)	VUR	[23]
MyD88	—	+	Mainly children	(Sweden)	VUR	[23]
SIGIRR	—	+	Mainly children	(Sweden)	VUR	[23]
TLR5	1174C>T	−	Adult women	Caucasian	UTM	[21]
TIRAP	589C>T	−	Adult women	Caucasian	UTM	[21]
	558C>T	−				
VEGF	460T>C	−	Children	Korean	VUR	[24]
	405G>C	−				
TGF-β1	509C>T	−	Children	Korean	VUR	[24]
	800G>A	−				
	869T>C	−				

higher in patients with recurrent UTI than in control groups. In particular, the OR of an HSPA1B 1267A>G polymorphism was 3 times higher in patients with recurrent UTIs. The study included patients with VUR of varied severity. No information is available on patients without VUR for both HSPAIB and CXCR1 polymorphisms [17, 19]. The OR of the TLR1 1805G>T gene polymorphism was lower in patients with pyelonephritis and comparable to those with cystitis than in controls. This population study included patients with urinary tract malformations [21]. The OR of TLR2 2258A allele was 3.1 times higher in children with recurrent UTIs, without VUR, than in controls [22]. Moreover, the OR of TLR2 2258A allele was not more significant in adult women with UTIs, including those with UTM, than in controls [21]. The frequency and OR of the TLR4 896A>G gene polymorphism were lower in patients with recurrent cystitis than in controls. The present study included patients and controls with urinary tract malformations [21]. Moreover, the frequency of a TLR4 896A>G gene polymorphism was slightly lower although statistically comparable to patients without VUR and controls. On the contrary, the OR was twice as high in patients, including those with VUR, than in controls [17]. The OR of the TLR4 1196C>T polymorphism was lower in patients with recurrent cystitis than in controls and comparable to patients with APN and controls. The TLR5 1174C>T polymorphism was higher in patients with recurrent cystitis and comparable to patients with APN and controls, including patients with UTMs [21]. The frequency of the VEGF 460T>C polymorphism was higher in patients with UTI, including those with VUR, than in controls. Therefore, the frequency was comparable in patients without VUR but not with controls. Furthermore, the frequency of TGF-β1 -509C>T and -800G>A polymorphisms were lower in patients with UTI, including those with VUR, than in controls. The frequency was comparable between patients with UTI, without VUR, but not with controls [24].

Table 2: Comparison of the genotype frequencies for each polymorphism studied in patients with recurrent urinary tract infection and in controls. (CXCR: IL-8 receptor; HSPA1B: Heat shock protein 72; NS: not significant; PS: pyelonephritis; TGF: Transforming growth factor; TIRAP: TIR domain-containing adapter protein; TLR: Toll-like receptor; UTM: urinary tract malformations; VEGF: Vascular endothelial growth factor).

Gene	Polymorphisms	Urological condition also included (infection type)	Patients/controls (N/N)	Risk allele	OR (95% CI)	*P* value	References
HSPA1B	1267A>G	VUR	103/235	1267G	3.37 (1.04 –10.93)	**.036**	[17]
CXCR1	217C>G	VUR, (Pyelonephritis)	60/226	217G	—	**.0007**	[18]
	2608G>C	VUR, (Pyelonephritis)	60/226	2608C	—	**<.001**	
TLR1	1805G>T	UTM, (Pyelonephritis)	281/286	1805G	0.53 (0.29–0.96)	**.035**	[21]
		UTM, (Recurrent cystitis)	303/286	—	0.73 (0.46–1.17)	NS	
TLR2	2258G>A	—	124/116	2258A	3.14 (1.53–6.44)	**.001**	[22]
		UTM, (Pyelonephritis)	329/316	—	0.93 (0.48–1.81)	NS	[21]
		UTM, (Recurrent cystitis)	337/316	—	0.83 (0.42–1.63)	NS	
TLR4	896A>G	UTM, (Pyelonephritis)	318/313	—	1.10 (0.69–1.75)	NS	[21]
		UTM, (Recurrent cystitis)	334/313	896A	0.54 (0.32–0.93)	**.025**	
		VUR	103/235	896G	2.19 (1.05– 4.57)	**.034**	[17]
		—	53/235	—	0.33 (0.10–1.12)	.067	
	1196C>T	UTM, (Pyelonephritis)	320/316	—	1.10 (0.69–1.75)	NS	[21]
		UTM, (Recurrent cystitis)	338/316	1196C	0.59 (0.35–0.998)	**.049**	
TRIF	9T>9	VUR	12/11	—	—	NS	[23]
TLR5	1174C>T	UTM, (Pyelonephritis)	320/315	—	1.17 (0.17–2.09)	NS	[21]
		UTM, (Recurrent cystitis)	337/315	1174T	1.81 (1.00–3.08)	**.030**	
TIRAP	539C>T	UTM, (Pyelonephritis)	318/314	—	0.86 (0.61–1.23)	NS	[21]
		UTM, (Recurrent cystitis)	338/314	—	0.88 (0.62–1.24)	NS	
	558C>T	UTM, (Pyelonephritis)	317/313	—	1.06 (0.77, 1.46)	NS	
		UTM, (Recurrent cystitis)	329/313	—	1.30 (0.95, 1.78)	NS	
VEGF	460T>C	VUR	63/71	460C	—	**<.05**	[24]
		—	18/71	—		NS	
	405G>C	VUR	71/82	—	—	NS	
		—	19/82	—		NS	
TGF-β1	-509C>T	VUR	78/80	-509C	—	**<.05**	[24]
		—	21/80	—		NS	
	-800G>A	VUR	79/89	-800G	—	**<.05**	
		—	22/89	—		NS	
	869T>C	VUR	77/85	—	—	NS	
		—	20/85	—		NS	

Table 3 summarizes the studies concerning protein expression in patients with susceptibility to UTI. Patients with recurring UTIs showed lower expression levels of CXCR1 than control patients did. The study included patients with VUR of various degrees [13, 16, 18, 19]. Smithson et al., investigating premenopausal women with recurrent UTI but no VUR, showed that CXCR1 expression was comparable to controls. However, women had low expression levels of CXCR2 [20]. Ragnarsdattir et al. compared TLR4 and the TLR4 adaptor proteins TRIF and SIGIRR genes in children with asymptomatic bacteriurias, including those with VUR, with controls. The TLR4 and SIGIRR genes had reduced gene expression, whereas the TRIF gene had increased expression [23].

3.6. Risk of Bias in Included Studies. Table 4 summarizes the potential risks of bias for each study. One study completed an analysis of both genetic polymorphisms and expression of the CXCR1 gene [18]. Another study performed the expression of the TRIF gene, but the frequency of the TRIF polymorphism was performed in only one quote of these patients (71% patients versus 46% controls) [23]. One study assessed the CXCR1 expression, but the frequency of the CXCR1 gene polymorphism was not estimated in the control individuals [20]. Ethnicity was reported in five out of nine studies [17, 18, 21, 22, 24]. Hardy-Weinberg equilibrium was assessed in 3 out of 5 studies [17, 21, 22]. In four studies, this assessment was not applicable [16, 18, 20, 23]. One study, investigating for TGF-β1 and VEGF polymorphisms,

Table 3: Comparison of the gene expression of patients with recurrent urinary tract infection and controls. (CXCR: IL-8 receptors; MyD88: myeloid differentiation primary response gene 88; SIGIRR: single immunoglobulin domain-containing IL-1 receptor–related molecule; TLR: Toll-like receptor; TRAM: TIR domain–containing adaptor inducing IFN-β-related adaptor molecule; TRIF: TIR domain–containing adaptor inducing INF-β).

Protein expression	Patients/controls (N/N)	Patient values (range or ± SD)	Control value (range or ± SD)	P	References
CXCR1	12/12	0.33 ± 0.23	1.33 ± 0.64	**<.05**	[13, 16]
	60/226	−1.44 [−6.52–(1.04)]*	0.28 [−0.73–(2.15)]*	**<.0001**	[18]
	10/15	70% (42%–96%)	100% (77%–165%)	**.0006**	[19]
	20/30	118.9 (102.7–158.9) AU	116.2 (103.7–139.5) AU	NS	[20]
CXCR2	20/30	14.9 (12.82–17.23) AU	18.9 (16.2–23.6) AU	**.002**	[20]
TLR4	17/24	33.2 (4.3–74.3)	56.1 (19.2–87.6)	**.003**	[23]
MyD88		100.5 (9.3–197.3)	84.3 (9.9–168.5)	NS	
TRIF		139 (34.5–315.1)	85.1 (12.4–171.5)	**.02**	
TRAM		107.9 (32–262.9)	101.7 (39.8–269.2)	NS	
SIGIRR		14.9 (8.2–63.3)	21.9 (12.5–35.5)	**.0005**	

Legend :* : compared to a standard; AU: arbitrary unit.

did not clarify whether the patient had recurrent UTIs although its objective was to assess susceptibility to UTIs. These patients were enrolled at their first UTI [24]. One study concerning TLR4 gene expression investigated for primary and secondary bacteriurias [23]. In only one study, concerning the TLR2 gene polymorphism, patients had no urological malformations [22]. Two studies concerning the HSPA1B, TLR4, VEGF, and TGF-β1 gene polymorphisms performed the statistical analysis in a subgroup of patients without VUR [17, 24].

4. Discussion

The first protection against a UTI involves physical barriers (unidirectional urinary flow), epithelial cells and the production of proteins that hinder bacteria adhesion [38]. The structural variability of host cell glycoconjugates characterizes pathogen recognition at mucosal sites. Subsequently, the oncoming inflammation activates the uroepitelial cells, which produce mediators of inflammation, until the pathogens are destroyed and eliminated. The functional chemokines and chemokine receptors are crucial for neutrophil recruitment and for neutrophil dependent bacterial clearance. Moreover, the neutrophil-mediated defense is essential for resistance to UTIs.

The inter-individual variability in cellular response may be responsible for variable clinical outcomes and may explain why certain individuals are susceptible to recurrent infections [38]. Susceptibility to UTIs is caused by inefficient bacterial clearance due to an impairment of the innate host defense, such as low chemokine receptor expression [16]. The neutrophils' functional state is influenced by the chemokine receptor expression of the urinary tract. Thus, the neutrophil and local environment of cytokine/chemokine/cell adhesion molecules, which govern the intensity and persistence of the host response, have been actively investigated [39].

Six out of 14 genes investigated in humans (HSPA1B, CXCR1 and 2, TLR1,2,4,5, SIGIRR, TRIF, TRAM, MyD88 TIRAP, VEGF, and TGF-1β) may be associated with susceptibility to UTI recurrence in humans. In particular, the HSPA1B, CXCR1 & 2, TLR2, TLR4 and TGF-β1 genes seem to be associated at various levels with an alteration of the host response to the UTI. The HSPA1B gene encodes a 70 kDa heat shock protein (HSP) that is a member of the HSP70 family [17]. Patients with recurrent UTIs showed a high prevalence of the HSPA1B 1267G allele [17]. The CXCR1 gene encodes a human chemokine receptor gene for IL-8 chemokine [18]. Mutational inactivation of the CXCR1 causes both acute disease and chronic tissue damage in mice [40]. Cell surface CXCR1 expression and CXCR1-specific mRNA levels were low in the neutrophils of both mice and children with recurrent UTIs [16]. Pyelonephritis prone children showed low expression levels of CXCR1 [23]. Patients susceptible to UTIs after the age of 15 years showed CXCR1 gene expression comparable to controls [20]. However, humans with recurrent UTI showed a high frequency of polymorphic allele (217G and 2608C) as well as reduced gene expression [18]. Moreover, CXCR2 expression in children aged 2–12 years at first manifestation of a UTI was comparable to controls [13]; whereas premenopausal women with recurrent UTI had lower CXCR2 expression than age-matched controls [20]. These data seem to suggest that the CXCR1 and CXCR2 genes could be associated with early or late manifestation of susceptibility, respectively.

TLRs are a family of receptors that recognize pathogen-associated molecules, and whose activation leads to the transcription of appropriate host-defense genes and to the recruitment of leucocytes [21]. Tabel et al. showed that TLR2 2258A allele in children has been implicated in susceptibility to gram-positive pathogens [22], but not in adult women with UTI including those with UTMs [21]. However, genetic alteration of TLR4 signaling could modify the host unresponsiveness to a UTI towards asymptomatic bacteriuria (ABU) rather than symptomatic pyelonephritis [41]. Children with asymptomatic bacteriuria expressed less

Table 4: Methodological quality of each included study. The studies are listed according to their presentation along this review. (CXCR: IL-8 receptor; MyD88: myeloid differentiation primary response gene 88; NA: not applicable; SIGIRR: single immunoglobulin domain-containing IL-1 receptor-related molecule; TLR: Toll-like receptor; TRAM: TIR domain–containing adaptor inducing IFN-β–related adaptor molecule; TRIF: TIR domain-containing adaptor inducing INF-β).

Authors	Gene polymorphism(s)	Protein(s) expression	Ethnicity	Hardy-Weinberg equilibrium	UTI recurrence	Exclusion of urinary tract malformations
Lundstedt et al. [18]	CXCR1	CXCR1	Caucasians	–	+	–
Smithson et al. [20]	CXCR1 [Unavailable controls]	CXCR1, CXCR2	—	NA	+	+
Lundstedt et al. [19]	—	CXCR1	—	NA	+	–
Frendéus et al. [13, 16]	—	CXCR1	—	NA	+	–
Hawn et al. [21]	TLR1, TLR2,TLR4, TLR5, TIRAP	—	Caucasian	+	+	–
Tabel et al. [22]	TLR2	—	Turkish	+	+	–
Karoly et al. [17]	TLR4, HSPA1B	—	Hungarian	+	+	–
Ragnardosttir et al. [23]	TRIF (assessed in a proportion of patients)	TLR4, MyD88, TRIF, TRAM	—	NA	[asymptomatic bacteriurias]	–
Yim et al. [24]	VEGF, TGF-β1	—	Korean	–	–	–

TLR4 than age-matched controls [23]. However, the TLR4 gene showed comparable frequency of allele and genotype [17, 21]. Thus, the authors extended the investigation to TLR4 regulatory genes. In particular, the TRIF gene showed increased expression and SIGIRR at reduced expression levels [23]. Moreover, TGF-β1 -509T allele showed a protective role in predisposition to recurrent UTIs because they were less frequent in children with recurrent UTIs. TGF-β1 appears to be a key cytokine involved in the regulation of cell proliferation, differentiation, extracellular matrix formation and immune response [24].

The available studies are heterogeneous for population study and thus difficult to compare with each other; they studied different ethnic groups, included patients with urinary tract malformations of various types and degree. They are limited in number which makes a meta-analytical study impossible. However, the assessment of protein expression was not performed for most of the genes studied. Therefore, CXCR1 is the most important gene that seems to be involved in susceptibility to recurrent UTI. A reduced CXCR1 expression was observed in both children and adults with recurrent UTI. It is likely that these studies on the CXCR1 gene may be biased because of inclusion of patients with VUR [16, 18]. Other genes could be associated with UTI susceptibility: polymorphisms of HSPA1B giving susceptibility [17] and TGF-1 β giving protection [24], although these studies could be biased by inclusion of patients with VUR. Limited information is available on CXCR2, TLR2 and TLR4 pathways to arrive at any conclusion on UTI susceptibility in humans. However, TLR4 expression was reduced in children with recurrent UTIs [23], whereas its 1196C>T polymorphism was shown to be protective in adult women with recurrent cystitis [21]. These studies included patients with UTMs. The frequency of HSPA1B (1267A>G), CXCR1 (217C>G, 2608G>C), TLR4 (1196C>T), TLR5 (1174C>T), VEGF (460T>C) and TGF-β1 (-509C>T and -800G>A) polymorphisms were different between patients with recurrent UTI and controls, but the study population included subjects with UTMs. In this context, TGF-β1 protected from UTI recurrences in patients with VUR. Finally, only three studies, investigating TLR2, TLR4, VEGF, and TGF β1 polymorphisms, were conducted on patients without VUR [17, 21, 24]; all but one (TLR2 2258G>A in children) showed no significant associations. In particular, the frequency of TLR2 2258A allele was high in children susceptible to UTIs [22]. These data have not been recently confirmed in adult women with UTI, including individuals with UTM [21]. The inclusion of patients of different ages and the presence of UTM make it impossible to make a comparison between the two studies and offer conclusive remarks.

In conclusion, the pathophysiological background of susceptibility to recurrent UTIs remains to be completely clarified, taking into account that multiple factors, such as gene-gene and gene-environmental interactions could be involved. The kidney may act as a host where clusters of bacteria persist as quiescent reservoirs surviving the immune response and resisting antibiotic attacks. Moreover, recent findings suggest that the genome of bacterial strains are associated with either asymptomatic, commensal-like strains that cause asymptomatic bacteriurias or with symptomatic infections [42], suggesting that the simple colonization or an infection can also be related to an interaction between the bacterial gene-patient gene relationship. Recent advances have suggested that a deregulation of candidate genes in humans could possibly predispose patients to recurrent UTI. The recognition of a genetic component in UTI recurrences will make it possible to diagnose at-risk adults and to predict genetic recurrences in their offspring. Marked differences in APN-associated morbidity between relatives of APN-prone

patients and the relatives of control subjects suggested that inheritance could be autosomal involving one or more genes [18]. For example, the combinations of polymorphisms of CXCR1 and TGF-β1 in the same individual could have additional effects on susceptibility. The treatment options with prophylactic antibiotics to prevent recurrent UTIs in children remain of doubtful usefulness [43]. The identification of susceptible patients could enable novel strategies in the management of recurrent UTIs. Antagonists and agonists of candidate gene products could manipulate host defense mechanisms, offering new therapeutic options to physicians treating UTI [44].

Abbreviations

APN: Acute pyelonephritis
UTI: Urinary tract infection
UTM: Urinary tract malformation.

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Research Article

Gene Expression Profiling of Placentas Affected by Pre-Eclampsia

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Several studies point to the placenta as the primary cause of pre-eclampsia. Our objective was to identify placental genes that may contribute to the development of pre-eclampsia. RNA was purified from tissue biopsies from eleven pre-eclamptic placentas and eighteen normal controls. Messenger RNA expression from pooled samples was analysed by microarrays. Verification of the expression of selected genes was performed using real-time PCR. A surprisingly low number of genes (21 out of 15,000) were identified as differentially expressed. Among these were genes not previously associated with pre-eclampsia as bradykinin B1 receptor and a 14-3-3 protein, but also genes that have already been connected with pre-eclampsia, for example, inhibin beta A subunit and leptin. A low number of genes were repeatedly identified as differentially expressed, because they may represent the endpoint of a cascade of events effectuated throughout gestation. They were associated with transcriptional regulation and vasoregulative pathways, along with a number of hypothetical proteins and gene sequences with unknown functions.

1. Introduction

One of the leading causes of mortality and morbidity amongst pregnant women and their offspring is pre-eclampsia (PE), affecting 2%–8% of all pregnancies depending on the criteria of diagnosis and ethnic population [1]. Pre-eclampsia is defined by hypertension and proteinuria in the pregnant woman. The symptoms manifest themselves in the late second or third trimester of pregnancy but there is most likely a long subclinical course of events before the debut of symptoms [2]. A rare complication of the condition is eclampsia involving encephalopathia and seizures, sometimes with a fatal result. For the fetus, pre-eclampsia commonly leads to intrauterine growth retardation (IUGR), low birth weight, prematurity, or perinatal death [3]. In early pregnancy cytotrophoblast cells invade the placental bed, resulting in a physiological remodelling of the spiral arteries. During the normal non-pre-eclamptic invasion process, the trophoblast becomes embedded in the vessel wall and the muscular and elastic components are disrupted, transforming the arteries into dilated low resistance vessels. These vessels are unable to constrict in response to vasoactive stimuli thereby ensuring an adequate blood supply to the placenta and the fetus. In pre-eclampsia, the trophoblastic invasion is impaired and the spiral arteries retain their endothelial lining and musculature, compromising blood flow into the intervillous space and thereby restricting the blood supply to the placenta and the fetus giving rise to ischemic hypoxia and oxidative stress [4–6].

The cause of pre-eclampsia is unknown, but it is linked to the presence of a placenta, since pre-eclampsia can occur in molar pregnancies where a placenta, but no fetus, is present [7]. Also, the only effective treatment of pre-eclampsia is delivery of the placenta. Studies have shown that some families are predisposed for development of pre-eclampsia and it has therefore been concluded that a hereditary element exists [8, 9], most likely to be the result of an interaction between several different genes.

In an attempt to identify genes that may be of importance for the pathological and pathophysiological changes seen in the pre-eclamptic placentas and to identify pre-eclamptic factors, which may be released into the maternal blood circulation, we investigated the gene expression in term placentas from uncomplicated pregnancies and pregnancies

affected by pre-eclampsia and which may be involved in the endothelial dysfunction. Throughout this paper, we use the phrase "term" to designate the end of pregnancy by delivery at any time during gestation (rather than just full term) unless otherwise specified. All the placentas investigated are from the third trimester. It must therefore be remembered that the pathways which appear to be regulated in this study represent the endpoint of what must be presumed to be a cascade of events effectuated throughout gestation.

2. Materials and Methods

2.1. Study Population. This is a *case-control-study* consisting of pregnant women with pre-eclampsia and a control group with an uncomplicated pregnancy. The sole inclusion criteria used for the women suffering pre-eclampsia were a blood pressure ≥140/90 mmHg or a rise in diastolic blood pressure of ≥30 mmHg, proteinuria of ≥300 mg/24 hours or ≥300 mg/L or ≥1+ by urinary dipstick analysis. Personal or family history of PE was not recorded. All were self-reported nonsmokers. The project was in accordance with the Helsinki II declaration and approved by the local Ethics Committee. The women gave their informed consent prior to sampling. An overview of the clinical data of the included pregnant women is given in Table 1. The method of delivery differs between the cases and controls. Most cases relate to vaginal deliveries and most controls relate to elective Caesarean sections. This may have influenced the comparison of genes implicated in the induction and progression of labour since these genes may be differentially expressed due to the process of labour rather than the pathogenesis of PE. A number of additional control subjects that were not included in the array analysis were also analyzed by real-time PCR. The majority of these controls were multiparous women in whom, theoretically, a different placental gene expression may exist compared with that of primiparous women.

2.2. Tissue Samples. Placental biopsies were obtained from the maternal side of term placentas at a maximum of 30 minutes after delivery. Biopsies of approximately 0.2–0.5 cm^3 were taken from the center of the cotyledons evenly across the placenta. Some biopsies were transferred to 10 volumes of RNAlater solution (Ambion, Austin, Texas) and incubated at 4°C over night prior to freezing at −80°C. Others were immediately processed for RNA purification by lysis in Trizol (Invitrogen, Paisley, UK), followed by homogenisation and centrifugation to remove cellular debris. Hereafter, the lysate was frozen at −80°C. From a subset of the placentas, biopsies were snap-frozen in liquid nitrogen and stored at −80°C.

2.3. Purification of RNA from the Tissue Samples. Before analysis, the RNA was pooled into a total of six groups; three groups each consisting of equal amounts of RNA from three pre-eclamptic placentas and three groups each consisting of equal amounts of RNA from three matched normal controls (i.e., triplicate experiments a, b, and c with paired cases and controls, see Table 1). The placental samples were matched for maternal age, parity, gestational week at delivery, smoking habits, the gender of the child, and when possible, method of delivery (but as Table 1 shows this was not possible in all cases).

RNA was purified from two biopsies (each of 30 mg cut from the original larger biopsies) from each placenta using Trizol (Invitrogen) according to the instructions of the manufacturer. The RNA preparation was subjected to a clean-up procedure using RNeasy Mini Kit (QIAGEN, GmbH, Hilden, Germany) according to the instructions from the manufacturer. RNA concentrations were measured by spectrophotometer.

2.4. Gene Expression Analysis. The gene expression of the samples was analysed using GeneChip (HG-U133A, Affymetrix Inc., Santa Clara, CA, USA) microarrays according to the instructions of the manufacturer. In short, mRNA was reverse transcribed into first strand cDNA using a T7-Oligo(dT) Promoter primer (GenSet/Proligo, Paris, France), followed by a RNaseH-mediated second-strand cDNA synthesis. The double-stranded cDNA was purified and used as template in an in vitro transcription (IVT) reaction in the presence of biotin-labelled UTP and CTP (Enzo Diagnostics) to yield amplified and biotin-labelled cRNA (ENZO BioArray HighYield RNA Transcript Labelling Kit; Affymetrix Inc.). Biotin-labelled cRNA was cleaned, extracted, and fragmented randomly to approximately 250 bp and hybridized for 16 hours to an Affymetrix HG-U133A array, containing approximately 22,000 probe sets representing approximately 15,000 well substantiated genes. The arrays were washed, stained with phycoerytrin streptavidin (SAPE) using the Affymetrix Fluidics Station 400 and fluorescent image files were produced using the Affymetrix GeneArray scanner 2500.

Evaluation of the results was performed using the DNA-Chip Analyzer (dChip) programme, a freeware programme available at http://biosun1.harvard.edu/complab/dchip [10]. The image files (cel files) were imported into the software, and the array files were normalized using the multiarray invariant-set normalization method. All arrays in the experiment were normalized to the array with the median overall brightness, which was selected as the baseline. The invariant-set normalization is based on probe values belonging to nondifferentially expressed genes between the array being normalized and the baseline array (the invariant set). The invariant set is used to generate a piecewise linear median curve between the baseline probes and the experiment probes, which is used at the normalization curve.

M versus A (MVA) plots were constructed after sample normalization using the Affy package from the R, BioConductor (http://www.bioconductor.org) to check the correlation between the replicate samples in each group and the variation between the normal control group and PE group, respectively. In the MVA plots the A-axis plots the average log intensity (log chip1 + log chip2)/2 and the M-axis plots the log of ratio (chip1/chip2). A matrix of MVA plots was constructed for Control, Pre-eclampsia, and both sample groups, where the MVA plots are showed in the upper triangle and the IQR (interquartile range) of the Ms and

Table 1: Clinical data on the included women and deliveries.

Pre-eclampsia								Control						
Chip pool	Age	Para	Weeks of gestation	Sex of child	Smoking	Delivery	Co-morbidities	Chip pool	Age	Para	Weeks of gestation	Sex of child	Smoking	Delivery
A	26	I	39	M	N	IL		*A*	*27*	*I*	*39*	*M*	*N*	*ES*
A	*29*	*I*	*37*	*F*	*N*	*IL*		*A*	*35*	*I*	*38*	*F*	*N*	*ES*
A	***29***	*I*	*39*	*M*	*N*	*IL*		*A*	*35*	*I*	*38*	*M*	*N*	*ES*
B	32	I	33	M	N	ES		*B*	*33*	*I*	*35*	*M*	*N*	*IL+S*
B	25	I	39	F	N	IL	*	B	26	I	39	F	N	ES
B	26	I	40	M	N	IL		*B*	*28*	*I*	*39*	*M*	*N*	*ES*
C	24	I	38	F	N	V unc		C	32	I	39	F	N	V unc
C	29	II	37	F	N	V unc		C	30	II	39	F	N	ES
C	35	I	37	M	N	V unc		C	33	I	38	M	N	ES
—	29	I	32	M	N	AS	**							
—	24	I	36	M	N	AS								
								—	27	II	38	M	N	ES
								—	34	V	38	M	N	ES
								—	29	II	39	M	N	ES
								—	38	IV	38	F	N	ES
								—	36	III	37	M	N	ES
								—	25	I	39	F	N	ES
								—	35	II	38	M	N	ES
								—	31	II	38	M	N	ES
								—	33	II	38	F	N	AS

Italics mean no real-time PCR experiments performed. Paired pre-eclamptics and controls for microarray analysis are listed on the same line in the table. IL = induction of labour, ES = elective sectio, AS = acute section, V unc = uncomplicated vaginal delivery Co-morbidities: *abortus imminens, **possible HELLP.

the Median of the As are displayed in the lower triangle. A model-based expression index was calculated using perfect match signals only and comparisons between sample groups were performed. For all comparison analyses, the following parameters were used: Minimum fold change of 1.2 fold, minimum absolute change in signal intensity of 50 units and a *P*-value for the change in signal intensity of.05 or less. The dChip software calculates the upper and lower bounds of a 90 percent confidence interval for the fold change. We have filtered our results by the lower bound fold change, which is the more conservative estimate of the fold change. The triplicate pairs of samples were analyzed on six separate arrays. For analysis, the control arrays were grouped together and their mean signals were compared with the mean signals of the case arrays. From the resulting list of genes generated by dChip, we chose a number of genes to verify by real-time PCR.

2.5. Verification of Results from the Gene Expression Analysis by Real-Time PCR. A number of genes found to be differentially expressed between case and control placentas were examined by real-time PCR using a LightCycler instrument (Roche). The genes we focused on were fibulin 1A, inhibin, leptin, and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein. Templates for the verification were the RNA preparations from individual placentas. Nine RNA preparations originated from pre-eclamptic placentas and twelve preparations originated from control placentas. A minimum of two different biopsies for each placenta were included in the preparation. From the RNA preparations, cDNA was synthesized using Superscript II RNase H- Reverse Transcriptase (Invitrogen, CA, USA) according to the instructions of the manufacturer. Equal amounts of total RNA (1.7 μg) were used for all reverse transcription reactions. All cDNA templates were diluted 25× in water before analysis. All primers were used in a final concentration of 0.5 μM each and the $MgCl_2$ concentration was 2 mM for all reactions. Primers and PCR conditions for each gene are listed in Table 2. A varying number of touch-down cycles were performed before the PCR to improve specificity. The annealing temperature and the number of cycles for which it was used are listed in Table 2. All reactions were carried out in a total volume of 16 μL, using 1.6 μL of diluted template and 1.6 μL LightCycler FastStart Reaction Mix SYBR Green I (Roche) prepared according to the manufacturer's instructions. Standard curves were performed for each run to ensure the comparability of the samples. For generation of a standard curve of sample fluorescence against relative template concentration, we used a pool of equal amounts of all 22 individual cDNA preparations as template. Undiluted template pool and dilutions of 25×, 50×, and 100× were used to generate the standard curve. All the individual results were evaluated against this curve. Furthermore, GAPDH expression was determined for all samples and used for normalization before statistical analysis was carried out.

Table 2: Primers and PCR conditions.

Gene target	Forward primer 5′-3′	Reverse primer 5′-3′	TD[a] a °C/c	TD[a] b °C/c	TD[a] c °C/c	TD[a] d °C/c	PCR[b] °C/c
Fibulin 1A	aagttggcaggagtggagac	cccccataggtgaatcacag	58/2	57/2	56/2	—	55/35
sFLT-3	taagcacaccacgcccagtc	aagaccgcttgccagctacg	69/2	68/2	67/2	—	66/30
Inhibin	gcttcatgtgggcaaagtcg	ccccctttaagcccacttcc	64/2	62/	60/	59/2	58/30
Leptin	gtccaagctgtgcccatcc	cccaggctgtccaaggtctc	64/2	62/	60/	59/2	58/30

[(a)] Touchdown cycle, annealing temperature/number of cycles.
[(b)] PCR, annealing temperature/number of cycles.

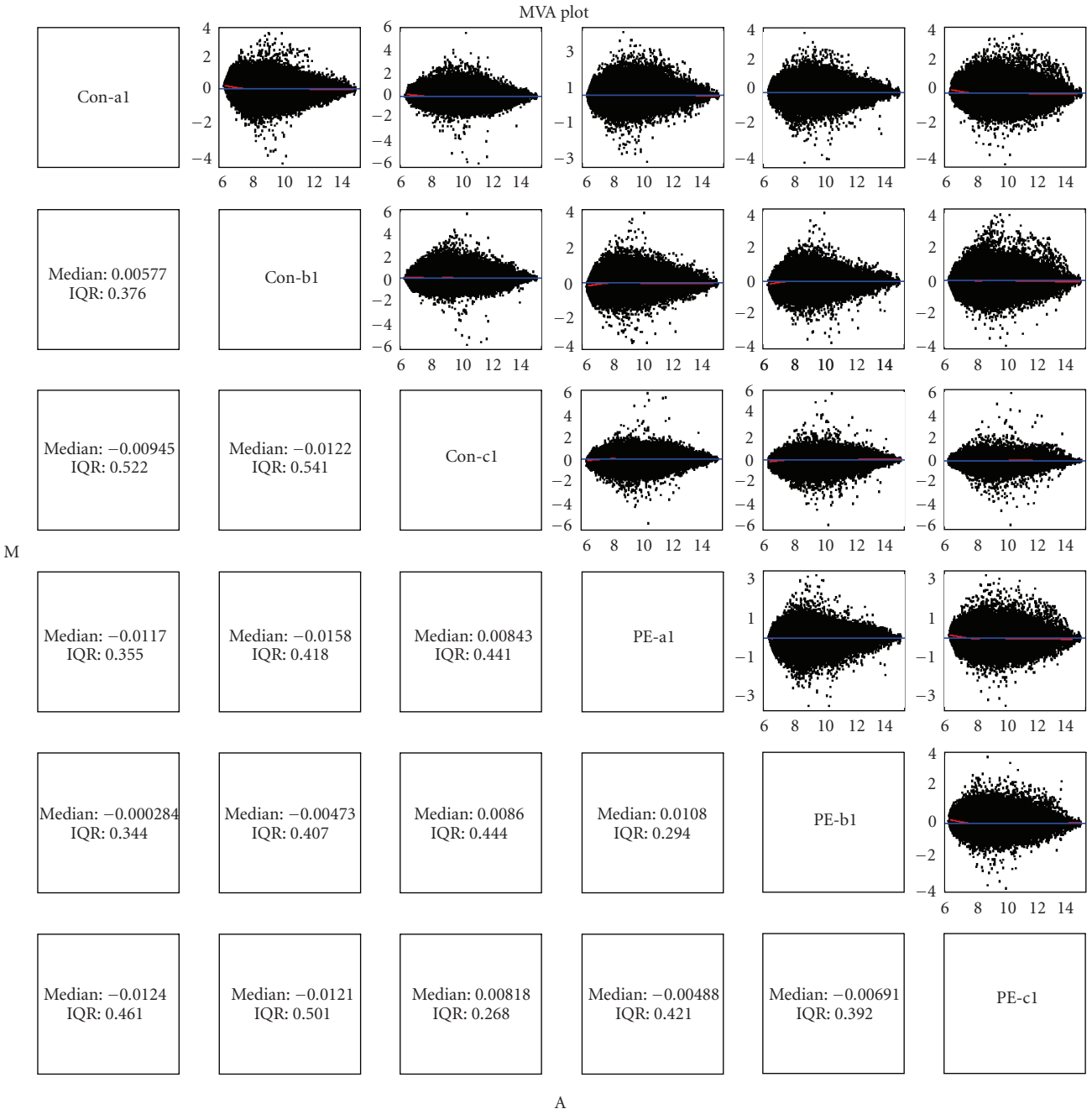

Figure 1: The interquartile range (IQR) values for the MVA plots show that the variation between the arrays within each group (case versus case or control versus control) is in the same range as the variation between the groups (case versus control). This indicates a high similarity between the case and control groups which is also apparent by the relatively low number of differentially expressed genes identified.

2.6. Statistical Analysis. The results generated from the real-time PCR experiments were compared using Mann-Whitney test.

3. Results

3.1. Microarray Data. A total of 21 genes were found to be differentially expressed between the arrays by dChip analysis, of these 9 were downregulated and 12 upregulated. The genes are listed in Table 3. A matrix of M versus A (MVA) plots was constructed for Controls, Pre-eclampsia and both sample groups (Figure 1). The interquartile range (IQR) values for the MVA plots showed that the variation between the arrays within each group (case versus case or control versus control) was in the same range as the variation between the groups (case versus control). This indicated a high similarity between the case and control groups, which is also apparent by the relatively low number of differentially expressed genes identified.

The genes chosen for real-time PCR verification were tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein and fibulin 1A on the basis of their fold change and their novelty in relation to the pathogenesis of PE. Inhibin and leptin have previously been associated with PE and were included as a verification of the PCR setup.

3.2. qRT-PCR Data. The relative expression levels calculated from the real-time PCR experiments are depicted in Figure 2. The regulation of fibulin between cases and controls observed on the arrays was not confirmed. The array analysis result was shown to have been influenced by a single sample expressing large amount of fibulin mRNA and the difference in expression observed by real-time PCR did not reach statistical significance ($P = .619$; FC 1.46). The differential expression indicated by the array analysis for leptin was verified ($P = .012$; FC 3.41). The difference in expression levels for inhibin and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein did not reach significance ($P = .500$, FC 1.13; $P = .166$, FC $-$ 1.27). The expression patterns for the genes examined by real-time PCR thus in general confirm the trends observed by microarray analysis.

4. Discussion

The most interesting feature of this study is the similarity between the arrays and the potential significance of the expression of 21 out of possible 15,000 genes. In hindsight that may not be very surprising. It is generally agreed that pre-eclampsia has a long subclinical course before the onset of symptoms and the main part of the causative placental molecular events that lead to the development of PE is temporary and may not be apparent at term. Also, tissues other than the placenta, such as the maternal vascular endothelium, may play a role in the pathogenesis of PE. This study includes cases of both late ($n = 9$) and early onset PE ($n = 2$) (cut-off 34 weeks of gestation). The pooled samples may mask any differences between the pathogenesis of late and early onset PE, but should on the other hand ensure that any gene expression differences detected is more likely a representative of the disease as a whole. We have also included true biological replicates in the current study design by pooling samples from other completely independent cases and controls; this is in contrast to several other published studies regarding gene expression in pre-eclamptic placentas, and should be a strength of the current study.

In the following, descriptions of the transcripts found to be differentially expressed by microarray analysis and/or further analysis are given and their relevance in relation to the pre-eclamptic syndrome is discussed. The sequences have been grouped in sections according to their possible influence on certain characteristics of pre-eclampsia: inadequate placentation, oxidative stress, inflammatory response, hypertension, and metabolism. Throughout the discussion, one should bear in mind that the results from this study rests on mRNA analysis only and that the mRNA expression profile of any biological system may not necessarily reflect the protein profile. Finally, a brief evaluation of the microarray experiment is given, followed by a general discussion, and a conclusion.

4.1. Gene Descriptions and Regulations

4.1.1. Inadequate Placentation. A complex of different thyroid hormone receptor-associated proteins (TRAPs) play a role as coactivators for thyroid hormone dependent transcriptional activation [11]. The placenta may be a thyroid hormone dependent tissue. Stimulation by thyroid hormones of trophoblast endocrine function resulting in enhanced production of human placental lactogen and human chorionic gonadotropin has been reported [12]. It has also been shown that L-triiodothyronine enhances the production of epidermal growth factor, a potent trophoblast mitogen [13]. Thyroid hormones may thus have an important role in villous development and placentation, in part mediated by the interaction between endocrine and autocrine factors [14]. We found a 240 kDa subunit (TRAP 240) expression decreased by 1.32 fold, which may indicate an impaired thyroid hormone signalling perhaps involved in the inadequate placentation observed in PE.

4.1.2. Oxidative Stress. The selenoprotein thioredoxin reductase (TrxR1) is a cytosolic antioxidant enzyme known to reduce many compounds in addition to thioredoxin, its principle protein substrate. It functions to eliminate reactive oxygen species (ROS) and regenerate oxidatively damaged proteins. Some have found that the levels of oxireductases, thioredoxin reductase included, were increased approximately 2- to 3-fold in pre-eclamptic placentas compared to normal placentas and it was speculated that this might be an adaptive response to the oxidative stress observed in PE [15]. The same group of investigators has also shown that oxidative stress does indeed induce thioredoxin reductase expression in the trophoblast [16]. However, we found thioredoxin reductase to be downregulated (1.57 fold) in the pre-eclamptic placentas compared with the normal controls. Failure to increase thioredoxin reductase may further promote a condition of oxidative stress.

Table 3: Regulation of genes in pre-eclamptic placentas compared to controls.

Accession[1]	Gene of interest	Fold change	*P*-value
Antioxidant enzymes			
NM_003330.1	thioredoxin reductase 1	−1.57	.025
Eicosanoid metabolism			
U63296.1	hydroxyprostaglandin dehydrogenase 15-(NAD^+),	1.48	.027
BC005939.1	prostaglandin D2 synthase 21 kDa (brain)	−1.51	.031
Energy metabolism			
AI761561	hexokinase 2	1.59	.045
NM_000230.1	leptin	2.94	.025
AA502643	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein e	−1.65	.005
Inhibin-activin system			
NM_002192.1	inhibin, beta A subunit (activin A, activin AB alpha polypeptide)	2.06	.007
Matrix proteins			
NM_000494.1	collagen, type XVII, alpha 1	1.50	.039
NM_006487.1	fibulin 1A	−2.49	.009
Transcription factors			
AA972711	zinc finger protein 292	−1.37	.024
NM_005121.1	thyroid hormone receptor-associated protein, 240 kDa subunit	−1.32	.012
Vascular factors			
NM_000710.1	bradykinin B1 receptor	−1.44	.024
NM_013332.1	hypoxia-inducible protein 2	1.61	.029
Miscellaneous			
AI206718	ESTs, Weakly similar to zinc finger protein 339	1.52	.024
AF052169.1	hypothetical protein BC013764	−1.46	.033
AF007149.1	hypothetical protein LOC257407	−1.40	.035
AK025495.1	KIAA0790 protein	1.56	.041
AK027231.1	KIAA1102 protein	1.54	.003
AB033025.1	KIAA1199 protein	1.59	.040
NM_004844.1	SH3-domain binding protein 5 (BTK-associated), (signal transduction)	1.58	.030
AF016535.1	ATP-binding cassette, sub-family B (MDR/TAP), member 1, (transporter activity)	1.45	.037

[1] Genbank accession number.

4.1.3. Inflammatory Response. Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon variant, is a so-called 14-3-3 protein. Interestingly, 14-3-3 proteins have also been found to exhibit antiapoptotic characteristics [17]. A decrease of 14-3-3, as identified by microarray analysis and real-time PCR, could therefore increase the occurrence of apoptosis in placental cells and thereby also increase the level of placental cellular debris shed into the maternal blood circulation. Syncytiotrophoblast microvillous membrane (STBM) fragments have been found at higher concentrations in the circulation of pre-eclamptic women compared to women with uncomplicated pregnancies [18]. It has been proposed that STBMs contribute to the vascular endothelial dysfunction of pre-eclamptic women.

4.1.4. Inhibin, Beta A Subunit. Activins and inhibins are growth and differentiation factors belonging to the transforming growth factor-beta superfamily. The activins are dimeric proteins consisting of two inhibin beta subunits (beta A homodimer or beta A beta B heterodimer). Either of the beta subunits in combination with an inhibin alpha subunit yield the protein inhibin, which has functions quite opposite of those of activin in that inhibin will inhibit the actions of activins. It has been shown that women with pre-eclampsia have increased serum levels of inhibin A and activin A [19]. Inhibin mRNA levels in pre-eclamptic placentas are elevated compared to normal controls [20] and activin A mRNA and protein are also increased [21, 22] suggesting that placental expression may contribute

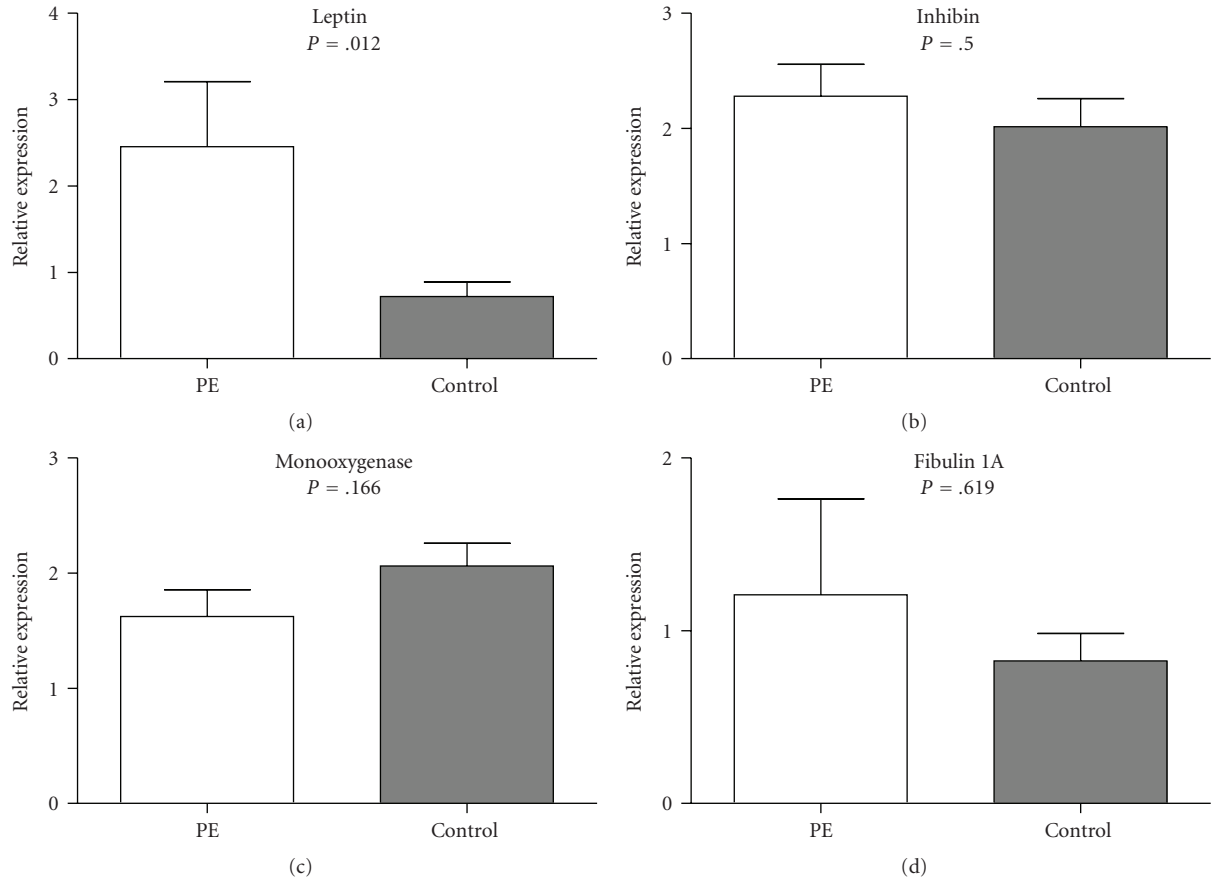

Figure 2: The expression patterns of the sequences as determined by real-time PCR. White bars represent pre-eclampsia cases; grey bars represent controls. Each bar represents the mean (+SEM) relative expression of the individual samples in the experiment. *P*-values for comparisons between cases and controls were: Fibulin (.619; FC 1.46), leptin (.012; FC 3.41), inhibin (.500; FC 1.13), tyr 3-monooxygenase/tryp 5-monooxygenase activation protein ε ($P = .166$; FC − 1.27); Mann-Whitney test. (Pre-eclampsia cases: $n = 9$, controls: $n = 12$; one control placenta was found to have a cDNA yield which was significantly lower than the other samples and it was therefore excluded from the subsequent analyses).

to the rise in serum concentrations, possibly more so for activin A than for inhibin A [22]. Activin A is also involved in inflammatory responses, though it seems to be dependent on dose whether the effect it exerts is anti- or proinflammatory [23]. Thus activin A may play a role in the maternal inflammatory response observed in pre-eclampsia. Microarray analysis indicated a 2.06-fold upregulation of inhibin beta A subunit, which was confirmed by real-time PCR (1.9-fold up-regulation). There is an increase of activin A levels in pre-eclampsia. Inhibin beta A subunit is part of activin A and may therefore directly contribute to the increased levels observed.

4.1.5. Hypertension. The eicosanoids such as prostaglandin and thromboxane are important mediators of blood vessel contractility and blood pressure regulation. We found two enzymes involved in the eicosanoid metabolism to be regulated between the pre-eclamptic placentas and the normal controls—hydroxyprostaglandin dehydrogenase 15 and prostaglandin D2 synthase. Hydroxyprostaglandin dehydrogenase 15 (15-PGDH, type I) is the main enzyme converting prostaglandins E2 and F2 to their biologically inactive 15-keto derivatives. Prostaglandin acts as a vasodilator. One study has found a twofold reduction of PGDH mRNA levels in placental tissue of patients with pre-eclampsia, compared with matched controls [24]. Others have found an increase in the activity of PGHD in pre-eclamptic placentas [25]. We found an upregulation (1.48-fold) of PGDH in the pre-eclamptic placentas. PGDH is involved in parturition and the expression is therefore dependent of the mode of delivery. During normal pregnancy at full term, PGDH transcription and activity in the chorio-decidua is reduced [26], allowing prostaglandins to reach the myometrium and to increase myometrial contractility. The placental expression does not seem to be affected [26]. In our study, a majority of the women in the PE group delivered vaginally, while almost all of the controls underwent elective Caesarean sections. Even taking this bias into account, the effect of parturition in the PE group should not be an increase of placental PGDH and it is therefore possible that PGDH plays a role in the pathogenesis of PE. Increased levels of PGDH would lead to decreased levels of biologically active vasodilatory

prostaglandins, which might contribute to a hypertensive state. Since results from different studies on the placental PGDH expression in PE are conflicting, further investigation is necessary.

Glutathione- (GSH-) independent PGD2 synthase catalyzes the conversion of prostaglandin H2 (PGH2) to PGD2 in the presence of various sulfhydryl compounds. One study showed that PGD2 synthase concentration in amniotic fluid is decreased in abnormal pregnancies [27]. It has been suggested that PGD2 might contribute to the maintenance of pregnancy by controlling the Th1/Th2 balance and antigen presentation by dendritic cells through a dual receptor system [28]. Prostaglandins are vasodilators and a down-regulation of prostaglandin D2 synthase as identified by our microarray analysis (1.51-fold) may contribute to the hypertensive state of PE. Its aberrant expression may also influence the reversal of the Th1/Th2 balance from that of normal pregnancies to that of pre-eclampsia.

Hypoxia-inducible protein 2 (hypoxia-inducible factor 2 alpha (HIF2A)) is a transcription factor, which is activated by hypoxic conditions. It is capable of specifically activating the transcription of the endothelial tyrosine kinase gene Tie-2 involved in angiogenesis [29]. Knock-out experiments in mice have shown that HIF2A also plays a role in the up-regulation of expression of the vasoconstrictors catecholamine and endothelin-1 [30, 31]. We found HIF2A mRNA expression up-regulated by 1.61-fold in PE. Others have previously found HIF2A protein, but not mRNA, expression upregulated in pre-eclamptic placentas [32]. The same investigators also found that villous explants from PE placentas fail to adequately downregulate hypoxia-induced HIF protein expression upon oxygenation [33].

Bradykinin B1 receptor is an inducible receptor, expressed during inflammatory conditions under the influence of cytokines. It mediates a vasorelaxing effect by inducing nitric oxide release. Bradykinin is a powerful angiogenic stimulus in vivo and in vitro. We found the expression of bradykinin receptor B1 in pre-eclamptic placentas to be 1.44-fold less than that in the normal controls, indicating that it might be involved in the development of hypertension and insufficient placental vascular growth associated with PE.

The leptin hormone is produced by adipose tissue and by the placenta during pregnancy [34]. In nonpregnant humans, leptin negatively regulates food intake and body weight via its receptor in the hypothalamus [34]. Leptin has also been shown to correlate with plasma insulin levels and an association between insulin resistance and resistance to leptin has been suggested [35]. In normal pregnancy, leptin levels increase, particularly in the second trimester, and decline postpartum [36]. Several studies have found leptin even further upregulated in pre-eclamptic pregnancies (see review by Poston [37]) while others have not been able to prove this association [38, 39] and one study has found that leptin levels were decreased in pre-eclampsia [40]. Some have suggested that upregulation of leptin in pre-eclampsia may cause maternal leptin resistance, which may be a component of insulin resistance, predisposing the onset of endothelial dysfunction [35]. An increase in maternal leptin levels may cause a rise in blood pressure through stimulation of sympathic activity, or it may have beneficial consequences, such as stimulation of fetal growth. It has been proposed that leptin controls the functional integrity of the feto-placental unit thereby maintaining pregnancy by virtue of its immunoregulatory property via T lymphocytes or proto-oncogenes [41]. We found leptin to be upregulated in the placenta in pre-eclamptic pregnancies. Others have also found leptin levels upregulated by microarray analysis [42, 43]. Our microarray analysis indicated an upregulation of 3.2-fold. Real-time PCR analysis yielded an up-regulation of approximately 32-fold. It shall be noted that such large differences in fold-change between microarray and corresponding PCR experiments are often observed.

4.1.6. Miscellaneous. A number of hypothetical proteins and gene sequences with unknown functions were also found to have expression levels that were markedly different in the pre-eclamptic group compared to the control group. Further investigation is needed before their relevance to the development of PE can be elucidated.

4.2. Microarray Analysis. The real-time PCR experiments in general exhibited the same trends of regulation that were evident from the array analyses. The risk of single samples biasing the overall results will always be a downside to analysis of pooled materials on microarrays and the importance of additional experiments to confirm the microarray results must be emphasized. Microarray studies of placental gene expression in pre-eclamptic placentas versus a control group data analysis with a specified set of parameters showed 59 genes [42], 96 genes [44], 137 genes [45], and 36 genes [45, 46] significantly differentially regulated between the two groups. Three of the genes exhibiting up-regulation are also present among the up-regulated genes identified in our study, namely, collagen XVII alpha 1, leptin, and inhibin beta A subunit. When comparing results from different case-control gene expression studies—microarrays, proteomic studies, or even gene-specific studies, several aspects must be taken into consideration. Firstly, differences in detection limits and analysis parameters may exist. Secondly, the design of the case and control groups will influence the results. The clinical characteristics, such as mean gestational age and parity of the included patients, will be of importance as well as the cellular composition of the placental samples. The mean gestational age of our study is higher (37.9 weeks for the samples studied on the microarrays) than that of the study of Reimer et al. [42] (mean gestational age 30.7 weeks) and unlike Reimer et al. we did not remove maternal decidual tissue from the placental biopsies. Our control group consists of women that have had uncomplicated pregnancies until delivery, the main part of which delivered by elective Caesarean section due to indications unrelated to the course of pregnancy. Finally, our study utilized the U133A chip, which represents sequences from ~15,000 genes.

The previous studies of gene expression in pre-eclamptic and control placental samples have revealed differences in the expression of immunologic factors. This was also observed in the microarray study by Reimer et al. [42]. For example,

Human Leukocyte Antigen-G (HLA-G) has been found to be differentially expressed in several studies. By relaxing the parameters of our data analysis (specifically by filtering the genes according to the upper bound value of the confidence interval for the fold changes), immunological genes such as T-cell receptor gamma-delta, killer cell immunoglobulin-like receptors, and HLA-G were also found to exhibit differences in expression between pre-eclampsia pools and controls. In some of the previously published studies, gestational ages were also shorter and other genes may be important in the pathogenesis of pre-eclampsia at these stages of pregnancy. So even though this study did not identify immunological factors as being differentially expressed, an immunological component in the pathogenesis of pre-eclampsia cannot be excluded for the first and second trimesters of pregnancy.

In conclusion, microarray analysis of the gene expression in pre-eclamptic placentas compared to that of normal controls indicated the regulation of a surprisingly small number of genes. However, the fact that others have found some of these genes regulated in connection with pre-eclampsia strengthens the credibility of the results of this study. Leptin and inhibin beta A subunit were both found to be up-regulated, which was confirmed by real-time PCR and which is consistent with the findings in other studies. These and other secreted factors may be of importance to the development of the maternal vascular endothelial dysfunction observed in pre-eclampsia. Apart from placental secreted proteins, other placental factors, perhaps including 14-3-3 proteins, working to increase the shedding of syncytiotrophoblast microvillous membranes (STBMs) into the maternal circulation may also contribute to this, since STBMs are found at higher concentration in the circulation of pre-eclamptic women compared to women with uncomplicated pregnancies [18]. Genes previously found to be connected with PE, such as thioredoxin reductase, hydroxyprostaglandin dehydrogenase, and hypoxia-inducible protein 2 alpha, were also identified by our array analysis. Furthermore, the microarray analysis yielded differential expression patterns between cases and controls for a number of novel factors not previously connected with PE, for example, prostaglandin D2 synthase and TRAP240. Surprisingly, no immunological factors were identified, but this may be because the pathways that we find regulated in this study represent the endpoint of what must be presumed to be a cascade of events effectuated throughout gestation. Early immune maladaptation may contribute to the shallow trophoblast invasion of the spiral arteries and the lack of spiral artery conversion into high-capacitance vessels observed in PE. This leads to a reduced placental perfusion and therefore a reduced nutrient supply and the generation of a hypoxic placental environment. Some of the genes identified in this study are hypoxia inducible transcription factors. Other genes implicated in gene transcription, such as additional transcription factors and proteins involved in growth factors signalling, were also found to be regulated. We hypothesize that factors induced by hypoxia or a reduced nutrient supply may contribute to an alteration of the regulation of gene expression in the pre-eclamptic placenta. Thus, some of the genes previously reported to be differentially expressed in pre-eclampsia may be so due to an aberrant regulation of expression rather than dysfunctional genes themselves. This, of course, does not mean that certain polymorphisms or mutations may not predispose to the development of PE and indeed several studies have shown associations between PE and genetic polymorphisms. These observations may partly explain the complexity of the disease and the vast number of genes [8] that have been implicated in the pathogenesis of pre-eclampsia up till now.

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